PHOSPHOLIPID SYNTHESIS AND FATTY ACID CONTENT OF PHOSPHOLIPIDS IN GERMINATING SEEDS AND SEEDLINGS OF CHILLING RESISTANT AND CHILLING SENSITIVE VEGETABLE SPECIES

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This is to certify that the

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Phospholipid Synthesis and Fatty Acid Content of Phospholipids in Germinating Seeds and Seedlings of Chilling Resistant and Chilling Sensitive Vegetable Species

presented by

Constantinos Christos Dogras

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ABSTRACT

PHOSPHOLIPID SYNTHESIS AND FATTY ACID CONTENT OF PHOSPHOLIPIDS IN GERMINATING SEEDS AND SEEDLINGS OF CHILLING RESISTANT AND CHILLING SENSITIVE VEGETABLE SPECIES

By

Constantinos Christos Dogras

Chilling sensitive plant species are injured by chilling temperatures, below about 12°C but above 0°C, while chilling resistant ones are not. Chilling injury has been related by some workers to a phase change in the lipid portion of cellular membranes and this has been correlated with the degree of saturation of those lipids.

In this study, the incorporation of ^{14}C-glycerol into phospholipids in germinating seeds and seedlings of chilling sensitive and resistant species, at 25°C and 10°C, has been investigated. In addition, the fatty acid content of the major phospholipids was determined.

Broad beans and peas, chilling resistant species, germinate at 10°C while the chilling sensitive lima beans do not. During seed imbibition at 10°C, a higher percentage phosphatidyl choline (PC) and a lower percentage phosphatidyl ethanolamine (PE) and phosphatidyl glycerol (PG) were observed in both axes and cotyledons of broad beans and peas

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than in lima beans. Furthermore, lima bean seeds, imbibed at 10°C, exhibited a lower percentage PC and a higher percentage PE and PG than at 25°C. The chilling resistant species, broad beans and peas, had about the same PC and PE at both temperatures.

Apparently, in the chilling sensitive lima beans at 10°C, there is a shift in metabolism towards the synthesis of more PG and PE largely at the expense of PC. A higher PC/PE ration, observed in broad beans and peas, may allow membranes to remain fluid and functional at low temperature (since PC remains liquid at lower temperatures than PE) while a lower ratio, observed in lima beans, may result in a phase change of the membrane lipids which would cause chilling injury and lack of seed germination.

In the seedling stage, there were no consistent differences between chilling resistant species (broad beans, peas, cabbage, beets, lettuce) and chilling sensitive ones (lima beans, watermelon, cucumber) in the relative percentages of individual phospholipids labeled.

In general, exposure of plants of chilling resistant species to 10°C continuously or shifting plants of chilling resistant and sensitive species from 25°C to 10°C caused an increase in percentage PC and a decrease in percentage PE in comparison to plants grown continuously at 25°C. These results suggest, that in the seedling stage of chilling sensitive species, a shift in metabolism occurs which

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produces more PC and less PE, similar to chilling resistant species, which allows them to survive exposure to chilling temperatures.

The fatty acid content of the three major phospholipids (phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl inositol) in germinating seeds of broad beans, peas, and lima beans revealed that the percentage 18:1 was higher and the percentage 18:3 was lower in the chilling resistant species than in lima beans. Also, over a 6-day germination period at 10°C in broad beans and peas, the percentage 18:1 decreased and the percentage 18:3 increased from the initial levels during imbibition. The fatty acid content of lima beans did not change during this 6-day period. This indicates that in chilling resistant plants, there is a shift in metabolism of fatty acids towards a more highly unsaturated state while in lima beans no shift occurs. This lack of a shift in fatty acid metabolism in lima beans may reflect the fact that these seeds were injured by the chilling temperature and no lipid metabolism occurred.

In the seedling stage, the chilling resistant plants had a higher percentage 18:2 and a lower percentage 18:3 than the chilling sensitive plants. In chilling resistant and sensitive species, with few exceptions, the percentage 18:3 increased after chilling hardening. The degree of unsaturation of fatty acids, as is reflected in the traditionally used indexes (double bond index and unsaturated

fatty acids/saturated fatty acids ratio), was not always positively correlated to the degree of chilling resistance of the species studied. The results of this study suggest that the chilling sensitivity of the species examined is related to the nature of cellular phospholipids, as characterized by the polar group of the molecule; furthermore, it is apparent that the fatty acid composition of phospholipids in chilling sensitive and resistant species is different, in terms of relative quantities of individual fatty acids.

PHOSPHOLIPID SYNTHESIS AND FATTY ACID CONTENT OF PHOSPHOLIPIDS IN GERMINATING SEEDS AND SEEDLINGS OF CHILLING RESISTANT AND CHILLING SENSITIVE VEGETABLE SPECIES

By

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INTRODUCTION

INTRODUCTION

Many plants, usually those originating in tropical or subtropical regions, are injured or fail to germinate and grow when they are exposed to chilling temperatures; that is, below about 12°C but above 0°C (Levitt, 1972; Lyons, 1973).

The damage caused by chilling temperatures is called chilling injury and is a time-temperature response, with the injury becoming more severe at lower temperatures or with longer exposure to the chilling temperatures (Lyons, 1973). Chilling resistance is determined by genetic factors but it is possible to partially condition or harden some chilling sensitive plants against chilling injury by exposing them, for a relatively short period of time, to temperatures slightly higher than the ones causing chilling injury (Wilson and Crawford, 1974; Wheaton and Morris, 1967). Investigations made so far support the theory of Lyons (1973) that upon chilling, a phase transition occurs in the membrane lipids of the chilling sensitive plant tissue, with concomitant changes in the physical properties of the membranes

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resulting in disruption of cell metabolism, ultimately leading to the observed chilling injury symptoms. Chilling resistant plants do not show the described phase change in membranes in the chilling temperature range. This suggests that membrane lipids of chilling resistant plants may be different from the ones of chilling sensitive plants and indeed some differences have been shown.

A literature review is following on the subject of the resistance of plants, and some other organisms, to chilling temperatures.

Literature Review

Lipids in Cell Membranes

It is believed that cellular lipids are mostly bound in membranes, with the exception of seed oils and leaf waxes (Kates, 1970). In all models of membranes, lipids are considered to be combined with structural proteins and a basic role in membrane structure and function is attributed to phospholipids (Van Deenen, 1965; Bishop, 1971).

Some investigators have proposed that the majority of the cell phospholipids are present in the membranes and it is speculated that only biological membranes contain phospholipids (Van Deenen, 1965; Chapman, 1967).

Subcellular organelles have been shown to be very rich in phospholipids (Van Deenen, 1965). During cell division an increased phospholipid synthesis is observed, ap

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apparently because phospholipids are necessary to build new membranes in the newly formed cells (Van Deenen, 1965).

Phospholipids, in addition to their structural role in membranes, are believed to be necessary for the function of certain enzymes associated with cell membranes (Cronan and Vagelos, 1972). The importance of the phospholipid composition of membranes in determining their permeability properties has been established in model and natural systems (Demel et al., 1972; Van Deenen, 1965; Williams and Chapman, 1970; Chapman and Leslie, 1970). There is a large variety of phospholipid classes associated with cell membranes (Chapman and Leslie, 1970; Kates, 1970).

In plants the major phospholipids are:* PI, PE, PC, PG and the minor ones are: PA, PS and CL (Kates, 1970).

The need for several phospholipid classes in biological membranes is not yet understood (Cronan and Vagelos, 1972), but it is suggested that the phospholipid variation may contribute to the several membrane functions. This idea is supported by the observation that a given biological membrane has a constant and characteristic lipid composition, suggesting that the physical properties of membrane lipids are essential for the function of membranes (Van Deenen, 1965).

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*PA: Phosphatidic Acid. PC: Phosphatidyl Choline. PE: Phosphatidyl Ethanolamine. PG: Phosphatidyl Glycerol. PI: Phosphatidyl Inositol. PS: Phosphatidyl Serine. CL: Cardiolipin. For chemical formulas see Appendix, Table A-1.

Furthermore it has been suggested that the presence of several phospholipid classes in biological membranes is necessary in order to produce the correct liquid cyrstalline phase organization required for membrane function (Williams and Chapman, 1970).

Physical Properties of Phospholipids in Relation to Temperature

Research with artificial membranes, prepared from chemically pure phospholipids, established that for each phospholipid a transition temperature (Tc) exists below which the phospholipid occurs in a crystalline form and above which it exists in a liquid-crystalline type of organization (Williams and Chapman, 1970; Chapman, 1967; Chapman and Leslie, 1970). The transition temperature is observed many degrees below the capillary melting point and depends on:

(1) The chain length of the hydrocarbons of the phospholipid; i.e., the shorter the chain length, the lower the Tc.

(2) The degree of unsaturation of the hydrocarbon chains; i.e., the more unsaturated the chains, the lower the Tc.

(3) The nature of the polar group of the phospholipid; i.e., PC exhibits lower Tc than PE when they have exactly the same kind of hydrocarbon chains (Appendix, Table A-3).

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(4) The amount of water present in the membrane system (the higher the water concentration, up to a limit, the lower the Tc).

(5) The amount and type of water soluble materials present.

(6) The presence of cholesterol.

It has been proposed (Williams and Chapman, 1970; Chapman, 1967), that the phase transition of phospholipids may be important in relation to the behavior of phospholipids in biological membranes at different temperatures. Furthermore it has been suggested (Chapman, 1967 and 1968; Cronan and Vagelos, 1972), that differing chain lengths and degrees of unsaturation of the fatty acids in the lipids of biological membranes allows the bio-membrane the correct fluidity, at a particular environmental temperature, in order to function properly.

Natural phospholipids extracted from mitochondria, glyoxysomes and proplastids of castor bean endosperm (Wade et al., 1974) exhibited a phase transition at about 10°C, below which chilling injury occurs for this species. The same phase transition, at the same temperature, was observed to occur in the whole organelles and their isolated membranes. A similar observation was made in mitochondrial lipids of the chilling sensitive sweet potato, while no phase change was observed in the chilling resistant potato at the chilling temperature of 12°C (Raison et al., 1971a). Phospholipids of Mycoplasma laidlawii exhibit a phase transition at lo

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low temperatures that is similar to the one observed in chemically pure phospholipids (Reinert and Steim, 1970). Egg yolk lecithin, a natural phosphatidyl choline, shows a marked endothermic phase transition at a temperature of about 20°C (Chapman, 1967).

The coincidence of a phase change occurring at low temperatures in chemically pure phospholipids, in natural phospholipids and in biological membranes collectively supports the idea that chilling injury may be related to physical changes occurring in the cell membranes.

Lipids and Cold Sensitivity in Organisms

The permeability of biological membranes is believed to be related to the fluidity of their lipids (Oldfield and Chapman, 1971). The length and degree of unsaturation of the hydrocarbon chains of the membrane lipids may determine the fluidity of the membrane at low temperature (Chapman, 1968; Bishop, 1971). It has been found (Lyons and Asmundson, 1965) that a small alteration (e.g., 5%) in the unsaturation of fatty acids in fatty acid mixtures, prepared from fatty acids similar to the ones occurring in biological membranes, results in a marked change of the freezing point of the mixture.

Mitochondrial lipids of chilling resistant plant species had more unsaturated fatty acids than the ones of chilling sensitive species (Lyons *et al.*, 1964). The mit

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mitochondria of the chilling resistant species were more flexible, a characteristic attributed to the higher degree of unsaturation.

On the other hand, it has been shown (Raison et al., 1971d) that the lipid components of the membranes of mitochondria from a chilling sensitive plant (sweet potato) undergo a thermal phase transition at about 12°C, where chilling injury occurs in many chilling sensitive species. No phase change was observed with mitochondria or the lipids extracted from mitochondria of a chilling resistant plant (potato) at that temperature. An increase in fatty acid unsaturation, in all lipid classes, in plants grown at low temperatures, has been shown in rye and wheat seedlings (Redshaw and Zalik, 1968; De La Roche et al., 1973) and alfalfa plants (Gerloff et al., 1966).

Membrane lipids in E. coli grown at lower temperatures were more unsaturated (Haest et al., 1969). Mitochondrial lipids of wheat seedlings (Miller et al., 1974) exhibited a lower Tc at low temperatures than at high ones, suggesting that some modification in the composition of lipids takes place at low temperatures.

Furthermore, it has been reported that some poikilothermic organisms at low temperatures, have more unsaturated phospholipids than their counterparts at higher temperatures (e.g., E. Coli, Cronan and Vagelos, 1972; Candida lipolytica, Kates and Paradis, 1973; Bacilus ce

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cereus, Kaneda, 1972; alfalfa, Grenier and Willemot, 1974; wheat plants, De La Roche et al., 1972). Cold resistant alfalfa varieties had more unsaturated phospholipids than cold sensitive ones (Grenier and Willemot, 1974).

Patterns of Phospholipid Synthesis in Relation to Temperature of Growth and Cold Resistance

More phospholipids, in comparison to other lipid classes, were synthesized in Populus euramericana, L. (Yoshida and Sakai, 1973) and Robinia pseudoacacia (Siminovitch et al., 1968) during the development of cold hardiness.

In some cases, the variation of the temperature or the development of cold hardiness resulted in the preferential accumulation of specific phospholipid classes. This may be important for the elucidation of the mechanism of cold resistance because different phospholipids show a phase transition from a liquid-crystalline to a solid-crystalline state at different temperatures as discussed earlier. In alfalfa (Kuiper, 1970), more PG and PI accumulated at high temperatures and more PC and PE at low ones. Cold resistant alfalfa varieties had more PC and PE at low temperatures than the cold sensitive ones. Also, in rye seedlings (Thomson and Zalik, 1973) more PI was synthesized at high temperatures than at low ones.

Smaller changes in phospholipids with temperature, were observed in wheat seedlings (Willemot, 1975) but clearly more PC was synthesized at low temperatures, especially in a cold resistant variety. Some workers, however, have shown no variation in the pattern of phospholipid synthesis with temperature or cold resistance changes as seen in *E. Coli* (Haest *et al.*, 1969; Cronan and Vagelos, 1972) and wheat seedlings (De La Roche, *et al.*, 1973).

Other factors may be involved in the determination of cold resistance in organisms, by altering the Tc of cellular lipids. For example, it has been suggested that cholesterol may affect the Tc of biological membranes (Lyons, 1973). This idea is supported by the finding that cholesterol lowers the Tc of phospholipids in model systems (Ladbrooke *et al.*, 1968).

Effect of the Physical State of Membrane Lipids on the Activity of Enzymes Associated with Them

Many of the enzymic reactions in cells take place on cellular membranes (Bishop, 1971). It has been demonstrated in chilling sensitive plants, that the activation energy for some enzyme systems, e.g. electron transport systems of both mitochondria and chloroplasts as well as protein synthesis systems, increases suddenly at chilling temperatures. This is suggestive of a conformational change in the membrane bound enzymes, probably because of a phase change observed to occur in the membrane lipids associated with the enzymes, at the chilling temperatures (Towers et al., 1972; Towers et al., 1973; Yamaki and Uritani, 1974; Shneyour et al., 1973; Raison, 1973; Raison et al., 1971b; Phillips and McWilliam, 1971; Lyons and Raison, 1970). In cold resistant species a similar sudden increase in the activation energy of the same membrane bound enzyme systems occurred at much lower temperatures than in the cold sensitive species. It is currently believed (Lyons, 1973) that at chilling temperatures the membrane lipids of the chilling sensitive species solidify and this causes an alteration of membrane permeability as well as an increase in the activation energy of some membrane bound enzymes. Both changes result in an imbalance of cell metabolism followed by the appearance of chilling injury symptoms.

Summary

(1) Cell membranes and their lipids are probably involved in the mechanims of chilling resistance in organisms.

(2) Phospholipids, major membrane lipids, may be important factors in chilling resistance. The hydrocarbon chain and polar groups in the phospholipid molecule probably contribute to the maintenance of a certain membrane fluidity at a particular temperature, allowing the membrane to function.

(3) Exposure of biological membranes to low temperatures presumably causes a physical change in their lipids from a liquid-crystalline to a solid-crystalline state. This results in some conformational changes of the enzymes associated with the lipids with an increase in activation energy and consequently an alteration in cell metabolism that may end in failure of growth and chilling injury.

(4) Chilling resistant or chilling hardened plants, apparently have (by inheritance or by modification in response to environmental temperature) membrane lipids that are subject to a phase change at lower temperatures than those of chilling sensitive species. This allows them to continue functioning normally at chilling temperatures (0-12°C).

Thesis Objectives

The discussion in the preceding pages shows that: (1) a specific relationship between temperature of growth or chilling resistance of plants and certain phospholipid classes, has not been established; (2) the knowledge on the fatty acid content of phospholipids of chilling sensitive, chilling resistant or chilling hardened plants is insufficient.

Because of the possible significance of phospholipids in the mechanism of chilling resistance and chilling injury investigations were initiated to determine: (1) the pa

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pattern of phospholipid synthesis, and (2) the fatty acid content of the major phospholipids (PI, PC, PE), in some chilling resistant, chilling sensitive and chilling hardened vegetable species under several temperature treatments.

The results of those studies are presented in the following four sections of this thesis.

SECTION I

PHOSPHOLIPID SYNTHESIS IN GERMINATING SEEDS OF SOME VEGETABLE SPECIES IN RELATION TO CHILLING RESISTANCE

PHOSPHOLIPID SYNTHESIS IN GERMINATING SEEDS OF SOME VEGETABLE SPECIES IN RELATION TO CHILLING RESISTANCE

Introduction

Chilling injury during the early stages of imbibition in germinating seeds of chilling sensitive species has been reported in many cases (Obendorf and Hobbs, 1970; Pollock and Toole, 1966; Pollock, 1969; Woodstock and Pollock, 1965; Greencia and Bramlage, 1971; Christianson, 1968). This injury results in inhibition of germination or in development of abnormal seedlings.

It has been shown, for many chilling sensitive plants exposed to chilling temperatures, that lipids of their cellular membranes are subject to a phase transition from a liquid to a solid gel state. This phase change has been correlated with a sudden increase of the activation energies of some enzyme systems associated with these membranes, resulting in the disruption of cell metabolism that is followed by the appearance of chilling injury symptoms

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(Towers et al., 1972 and 1973, Shneyour et al., 1973; Raison et al., 1971b; Lyons and Raison, 1970; Phillips and McWilliam, 1971).

Phospholipids are considered one of the most important lipid groups in biological membranes (Van Deene, 1965; Chapman, 1967; Bishop, 1971). It has been shown, that synthesized and natural phospholipids (Williams and Chapman, 1970; Chapman, 1967; Wade et al., 1974) exhibit a phase transition from a liquid-crystalline to a crystalline state at temperatures determined by, among other factors, the degree of unsaturation of the hydrocarbon groups and the nature of the polar group (Williams and Chapman, 1970; Chapman, 1967).

In some plant species, the pattern of phospholipid synthesis was found to be affected by the temperature of growth or the degree of cold resistance (Kuiper, 1970; Willemot, 1975; Thomson and Zalik, 1973). This suggests that a relationship may exist between specific classes of phospholipids and cold resistance. However, a clear relationship has not yet been established.

Lipid synthesis in many species has been observed to start during the early hours of seed imbibition (Hölzl and Wagner, 1971; Katayama and Funahashi, 1969; Shewry et al., 1973; Macher et al., 1975; Nawa and Asahi, 1971; Kagawa et al., 1973).

In this study an investigation was conducted on phospholipid synthesis during the imbibition stage of seed germination, in chilling resistant and chilling sensitive species, at chilling or non-chilling temperatures. The incorporation of ^{14}C-glycerol into phospholipids during the first 24 hours of seed imbibition was determined. The incorporation of ^{14}C-glycerol into phospholipids of germinating seeds, hazel and soybeans, has been demonstrated earlier by others (Stobart and Pinfield, 1970; Hölzl and Wagner, 1971).

The incorporation studies in this work were conducted in germinating seeds of the chilling resistant species *Vicia faba*, L., and *Pisum sativum*, L. as well as of the chilling sensitive *Phaseolus lunatus*, L. Germination studies with lima beans (Pollock and Toole, 1966; Woodstock and Pollock, 1965) have shown that this species is subject to chilling injury during the imbibition period when the temperature is below 15°C. The chilling resistance of the other two species examined has been well established (Robinson, 1968; Knott, 1962; Kotowski, 1926). Germination studies conducted with lima beans, broad beans and peas, at 10°C for a period of 25 days, revealed that lima beans did not germinate at that temperature while the other two species germinated and developed normal seedlings even though their growth was much slower at 10°C than at 25°C.

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Materials and Methods

Materials

Seeds of lima beans (*Phaseolus lunatus*, L., cv. Henderson bush) and peas (*Pisum sativum*, L., cv. Little Marvel) were purchased from Northrup, King & Co. (Fresno, California) seeds of broad beans (*Vicia faba* cv. Broad Windsor) were purchased from Stokes Seed Inc. (Buffalo, N.Y.). The seeds used were about one year old and of high germinability. Glycerol [$^{14}C(U)$] was purchased from New England Nuclear (Boston, Mass.). Precoated TLC plates (20x20 cm, 0.250 mm) were purchased from Analtech, Inc., (Newark, Delaware). Lipid standards were purchased from Applied Science Laboratories, Inc. (State College, Pennsylvania) and Supelco, Inc. (Bellefonte, Pennsylvania). Cab-O-Sil was purchased from Research Products International Corp. (Elk Grove Village, Illinois). All organic solvents used were distilled in glass and contained the antioxidant BHT (butylated hydroxytoluene) at 0.1mg/l (Turner and Rouser, 1970).

Methods

Seeds of lima beans, broad beans and peas were surface sterilized with 1% NaOCl and imbibed for 24 hours in the dark at 10°C or 25°C in sterilized water containing ^{14}C-glycerol (121 or 133.4 mCi/m mole at 0.2 μCi/ml). The

moisture content of the seeds was 10-11% on fresh weight basis. Three groups of seeds from each species were treated. Each group consisted of 20 seeds (lima beans and peas) and 12 seeds of broad beans. At the end of the imbibition, the seeds were frozen in liquid nitrogen and lyophilized, to avoid enzymatic degradation of lipids (Höelzl and Wagner, 1966; Thomson and Zalik, 1973). The lyophilized seeds were stored at -30°C until lipid extraction.

Lipid Extraction. The lyophilized seeds were separated into axes and cotyledons and ground to form a powder, using a glass homogenizer. The lipids were extracted from the ground tissue according to the method of Folch et al. (1957), by shaking with chloroform: methanol (C/M) (2:1 v/v) for 30-40 minutes at 30-35°C. The C/M supernatant was decanted and filtered through Whatman No. 41 filter paper. The residue was re-extracted two more times with C/M 2:1; the extracts were combined and washed with 0.2 volumes of 0.9% NaCl (Dawson et al., 1969) and shaken vigorously for two minutes. The mixture was allowed to settle and the upper phase was discarded while the lower one, containing the lipids was washed two more times with Folch's mixture (chloroform: methanol:water, 3:48:47 v/v) also containing 0.9% NaCl, to prevent partitioning of phospholipids to the upper phase wash. After the second washing, the lower, chloroform phase containing the lipids was evaporated to dryness under nitrogen, and the lipids were redissolved in chloroform for application to thin layer chromatography plates.

Thin Layer Chromatography (TLC): (1) Separation of phospholipids: The lipids extracted from the seeds were applied on precoated TLC plates (20x20 cm), coated with 0.250 mm Silica gel G (E. Merck, Darmstadt) layers, following activation of the layers at 110°C for 60 minutes. The phospholipids were separated from other lipids and from each other by 2 dimensional thin layer chromatography at about 25°C. The plates were developed in the first direction in chloroform/methanol/28% NH_4OH (65/25/4 v/v/v) and after drying for about 15-20 minutes, they were developed in the second direction in chloroform/methanol/acetic acid/water (85/15/10/3 v/v/v/v).

(2) Detection of phospholipids on the TLC plates: The chromatographed lipids were separated into the following phospholipids, among other lipid classes: (1) Major ones, containing more than 95% of the radioactivity counted in phospholipids: PI, PC, PE and PG, and (2) minor ones: PA, PS, CL and lysophosphatidyl choline (LPC). The phospholipids were identified by comparison of their migration on the TLC plates with that of pure standards and by using the following chromogenic reagents (Skipski and Barclay, 1969):

(a) Dragendorff's reagent for PC and LPC.

(b) Ninhydrin reagent for PE and PS.

(c) Periodate-Schiff reagent for PI and PG.

(d) Molybdenum blue reagent for all phospholipids. PI migrated close to PS and their separation was not complete but all the other phospholipids were completely separated.

(3) Measurement of radioactivity of phospholipids: The silica gel of the TLC plate, containing the labeled phospholipids, was scraped off the plate into scintilation vials, the scintilation liquid added, and the vials were shaken, for 4-5 hours to insure good distribution of the labeled phospholipids throughout the scintilation liquid. The scintilation liquid was made by mixing toluene with Triton-X-100 (2:1 v/v) and adding 4g of PPO (2,5-diphenyloxazole) and 0.1g of dimethyl POPOP (1,4-bis [2-(4-methyl-5-phenyloxazole)]-benzene) per liter of toluene (Patterson and Greene, 1965). Also included in the mixture was 3% Cab-O-Sil. The radioactivity of the phospholipids was measured in a Beckman LS-100 Liquid Scintilation Counter. The counting efficiency was about 95%. Corrections for quenching were made.

Results

In this experiment the label of ^{14}C-glycerol was incorporated primarily into PI, PC, PE and PG, representing more than 95% of the label distributed among all phospholipid, with the remaining label in PA, PS, CL and LPC (Appendix, Table A-4). The results will be presented as percentages of the CPM recorded for each phospholipid in relation to the total CPM of the four major phospholipids, i.e., $\frac{\text{CPM for PI}}{\text{CPM for (PI+PC+PE+PG)}} \times 100$ = percentage PI.

The results for each phospholipid will be discussed with reference to species, seed parts (axes or cotyledons) and temperature treatments in that order.

<u>Phosphatidyl Choline (PC)</u>

(1) Significant differences in the percentage PC between all three species at both temperatures were found in axes and cotyledons (Table 1, 2). Lima beans, with the exception of axis tissue at 25°C, had a significantly lower percentage PC than broad beans and peas (Tables 1 and 2). With the exception of lima bean cotyledons at 10°C, the incorporation of ^{14}C-glycerol into PC was higher than in any other phospholipid (Tables 1 and 2).

(2) When axes and cotyledons are compared at 10°C (Table 3) the cotyledons of broad beans and peas had a higher percentage PC, while in lima beans the axes had the higher percentage. At 25°C, broad bean cotyledons showed a higher percentage PC than axes, peas had the same percentage PC in both axes and cotyledons, while in lima beans, the axes had the higher percentage PC.

Table 1. Distribution of radioactivity (percentages) among phospholipids of seed axis tissue after a 24 hour imbibition with ^{14}C-glycerol at 10°C or 25°C.

Species (Axes)	Temperature °C	Phospholipid Percentages				L.S.D. (0.05)	PC/PE ratio	$\frac{\text{Phospholipid}}{\text{Total Lipid}} \times 100$
		PI	PC	PE	PG			
Broad Beans	10	7.29	56.95	25.66	10.06	2.07	2.218	53.38
Peas	10	10.70	57.96	21.56	9.74	1.95	2.693	46.07
Lima Beans	10	5.47	39.76	36.54	18.20	1.48	1.089	59.61
L.S.D. (0.05)		1.46	2.23	1.95	2.12		0.207	N.S.
Broad Beans	25	10.15	60.97	22.28	6.55	0.80	2.736	74.19
Peas	25	16.49	53.61	22.60	7.27	2.60	2.383	66.81
Lima Beans	25	7.83	57.33	28.58	6.56	1.33	2.006	65.71
L.S.D. (0.05)		1.80	1.62	2.17	N.S.		0.289	N.S.

Table 2. Distribution of radioactivity (percentages) among phospholipids of cotyledonary tissue after a 24 hour imbibition with ^{14}C-glycerol at 10°C or 25°C.

Species (Cotyledons)	Temperature °C	Phospholipid percentages				L.S.D. (0.05)	PC/PE ratio	$\frac{\text{Phospholipid}}{\text{Total Lipid}} \times 100$
		PI	PC	PE	PG			
Broad Beans	10	12.02	63.09	15.07	9.99	3.73	4.201	66.25
Peas	10	12.07	62.43	19.53	5.96	3.71	3.243	64.80
Lima Beans	10	8.42	25.24	35.57	30.61	3.34	0.708	53.99
L.S.D.(0.05)		N.S.	4.42	3.52	3.98		0.680	N.S.
Broad Beans	25	10.08	66.00	19.66	4.24	0.85	3.356	70.09
Peas	25	18.60	56.62	17.57	7.17	7.91	3.224	68.57
Lima Beans	25	15.32	48.59	30.42	5.57	1.59	1.600	70.45
L.S.D.(0.05)		3.69	3.67	1.22	0.75		0.240	N.S.

Table 3. Distribution of radioactivity (percentages) among seed phospholipids after a 24 hour imbibition with ^{14}C-glycerol at 10°C or 25°C. Comparison of temperatures and seed parts.

Phospholipids	Seed Part	Broad Beans			Peas			Lima Beans		
		10°C	25°C	L.S.D.(0.05)	10°C	25°C	L.S.D.(0.05)	10°C	25°C	L.S.D.(0.05)
PI	Axes	7.29	10.15	1.93	10.70	16.49	2.09	5.47	7.83	1.43
PI	Cotyledons	11.76	10.14	N.S.	12.07	18.80	5.37	8.42	15.32	1.89
L.S.D.(0.05)		N.S.	N.S.		N.S.	N.S.		1.02	2.18	
PC	Axes	56.95	60.97	2.25	57.86	53.61	2.61	39.76	57.33	1.69
PC	Cotyledons	63.09	66.00	N.S.	62.43	56.62	N.S.	25.24	48.66	2.74
L.S.D.(0.05)		4.31	1.06		4.64	N.S.		2.61	1.89	
PE	Axes	25.66	22.28	1.16	21.56	22.60	N.S.	36.54	28.58	2.22
PE	Cotyledons	15.07	19.66	2.30	19.53	17.57	N.S.	35.57	30.42	2.42
L.S.D.(0.05)		2.40	0.92		N.S.	2.72		N.S.	1.93	
PG	Axes	10.06	6.55	2.02	9.74	7.27	N.S.	18.20	6.22	1.32
PG	Cotyledons	9.99	4.24	2.85	5.96	7.17	0.84	30.61	5.57	4.83
L.S.D.(0.05)		N.S.	1.04		2.08	N.S.		4.92	N.S.	

(3) In cotyledons and axes of lima beans the percentage PC was higher at 25°C than 25 10°C, while in cotyledons of broad beans and peas no differences were observed with temperature (Table 3). However, in pea axes a higher percentage PC was found at 10°C while in broad bean axes a higher percentage PC was observed at 25°C than at 10°C (Table 3). It should be especially noted that the difference in percentage PC, between 25°C and 10°C, was quite large in lima beans (both axes and cotyledon) compared to differences in these two tissues of broad beans and peas (Table 3).

Phosphatidyl Ethanolamine (PE)

(1) Lima bean axes and cotyledons, at 10°C and 25°C, always had a higher percentage PE than the cold resistant species (Tables 1 and 2). In the axes of all species, at 10°C or 25°C, PE was the 2nd ranking phospholipid and the same was observed in cotyledons with the exception of lima beans at 10°C, where PE was the 1st ranking phospholipid (Table 1).

(2) No consistent differences in percentage PE between axes and cotyledons were detected in all three species (Table 3).

(3) A higher percentage PE was observed at 10°C than at 25°C in lima bean axes and cotyledons as well as in broad bean axes while no difference was observed in

peas (Table 3). In broad bean cotyledons more PE was synthesized at 25°C.

Phosphatidyl Inositol (PI)

(1) Significant differences observed between species in percentage PI of axis tissue, at 10°C and 25°C. Lima bean axes had a lower percentage PI at both temperatures than did axes from peas and broad beans (Table 1). Consistent differences between species were not noted in cotyledonary tissue (Table 2).

(2) Cotyledonary tissue of lima beans had a higher percentage PI at both temperatures than axis tissue. Peas and broad beans had the same percentage PI in cotyledons and axes (Table 3).

(3) In all three species, a higher percentage PI was observed at 25°C than at 10°C, with the exception of broad bean cotyledons which exhibited about the same amount of PI at both temperatures (Table 3).

Phosphatidyl Glycerol (PG)

(1) When species were compared, a significantly higher percentage PG was observed in lima beans at 10°C, in both axes and cotyledons (Tables 1 and 2). At 25°C no differences were observed between species in axis tissue while in cotyledons the highest percentage PG was observed in peas and the lowest one in broad beans (Tables 1 and 2).

(2) There were no consistent differences in percentage PG, in axes or cotyledons, between chilling sensitive lima beans and chilling resistant peas and broad beans (Table 3).

(3) In both axes and cotyledons of broad beans and lima beans a higher percentage PG was found at 10°C than at 25°C. No differences occurred with temperature in pea axes. A higher percentage PG was observed in pea cotyledons at 25°C than at 10°C (Table 3).

Phosphatidyl Choline / Phosphatidyl Ethanolamine Ratio

Because the transition temperature (Tc) of phospholipids is affected by the nature of the polar group (PC exhibits lower Tc than PE) and the fact that chilling sensitive lima bean axes and cotyledons at 10°C had a lower percentage PC and a higher percentage PE than chilling resistant peas and broad beans (Tables 1 and 2) the PC/PE ratio was investigated.

(1) Lima beans exhibited the lowest PC/PE ratio, at 10°C and 25°C, in both axes and cotyledons compared to broad beans and peas (Tables 1 and 2).

(2) When axes and cotyledons are compared (Table 4) the axes of lima beans, at both temperatures, had the higher ratio. In broad beans and peas, cotyledonary tissue had a higher or same ratio as the axis tissue.

Table 4. Comparison of radioactivity distributed (percentages) in phosphatidyl choline vs. phosphatidyl ethanolamine and in total phospholipids vs. total lipids after a 24 hour imbibition with ^{14}C-glycerol at 10°C or 25°C.

Phospholipid ratios	Seed Part	Broad Beans			Peas			Lima Beans		
		10°C	25°C	L.S.D.(0.05)	10°C	25°C	L.S.D.(0.05)	10°C	25°C	L.S.D.(0.05)
PC/PE	Axes	2.218	2.736	0.098	2.693	2.383	N.S.	1.089	2.006	0.139
PC/PE	Cotyledons	4.201	3.356	0.427	3.243	3.224	N.S.	0.708	1.600	0.115
L.S.D.(0.05)		0.422	0.117		N.S.	0.533		0.084	0.160	
$\frac{\text{Phospholipids} \times 100}{\text{Total Lipids}}$	Axes	53.38	74.19	5.57	46.07	66.81	9.11	59.61	65.71	N.S.
$\frac{\text{Phospholipids} \times 100}{\text{Total Lipids}}$	Cotyledons	66.25	70.09	N.S.	64.80	68.57	N.S.	53.99	70.45	11.38
L.S.D.(0.05)		5.21	N.S.		14.29	N.S.		N.S.	N.S.	

(3) In both axes and cotyledons of lima beans the PC/PE ratio was higher at 25°C than at 10°C (Table 4). In cotyledons and axes of peas the PC/PE ratio did not change with temperature. In broad bean axis the PC/PE was higher at 25°C than at 10°C but in cotyledons the ratio was higher at 10°C.

Phospholipids as Percent of Total Lipids

Since the quantity of phospholipids synthesized, in relation to other lipid classes, may reflect membrane building activity and because the growth of some plant species at low temperatures resulted in accumulation of phospholipids (Kuiper, 1970; De La Roche *et al.*, 1972) the percentage of phospholipids in relation to total lipids was calculated. In this study the following were observed:

(1) No significant differences were observed among species in the relative quantities of phospholipids synthesized (Tables 1 and 2).

(2) At 10°C, more phospholipids were synthesized in cotyledons than axes of the chilling resistant species (Table 4) while in lima beans no significant difference was observed between seed parts. At 25°C, in all species, no difference was observed between seed parts.

(3) Some differences were observed between temperatures but they were not consistent (Table 4).

Discussion

The *de novo* synthesis of phospholipids is essential for the production of new and for the repairing of old membranes (Van Deenen, 1965). During seed germination phospholipids are synthesized, presumably for use in the building of cellular and organellar membranes in the seedlings (Ching, 1972). The increase in mitochondrial phospholipids observed in pea cotyledons during imbibition (Nawa and Asahi, 1971) indicates synthesis of new or repair of old membranes. In this experiment, phospholipids were labeled in both axes and cotyledons of all species studied and it is assumed that *de novo* synthesis of phospholipids occurred since glycerol, that was used as the labeling source, is believed to be a precursor for synthesis of new phospholipid molecules (Mazliak, 1973). The results show that some of the phospholipids may be related to the chilling resistance of the studied species or to the temperature at which the seeds germinated.

The observation that PC/PE ratio at 10°C, was lower in the chilling sensitive lima beans than in the chilling resistant broad beans and peas suggests that PC and PE may be implicated in the determination of chilling resistance or cold sensitivity in these species.

It is known (Chapman, 1967; Williams and Chapman, 1970) that PC changes phase from liquid-crystalline to crystalline state at lower temperatures than PE and it has been suggested that this may be important for the function of biological membranes at different temperatures. Similar information is not available for PI and PG, however their capillary melting points are quite different: m.p. of PG is 67°C and of PI is 136°C (Williams and Chapman, 1970). The following are the most important conclusions from the data of this study:

(1) A higher percentage of radioactivity was found in PC in the chilling resistant species (broad beans and peas) than in the chilling sensitive lima beans at 10°C, especially in the cotyledonary tissue (Table 1). Furthermore, in lima beans, the percentage PC at 10°C was much lower than at 25°C in both axes and cotyledons (Table 3). This may suggest a shortage of available PC, at 10°C, for the building of new membranes or the repair of old ones leading to failure of germination and chilling injury. On the other hand, no significant differences or small differences were obserfed in percentage PC between temperatures in the chilling resistant species (Table 3). The above results suggest that PC may play an important role in the expression of chilling resistance in the species studied. The report concerning higher percentage PC in cold resistant alfalfa varieties (Kuiper, 1970) agrees with the results of this study of higher percent PC in chilling resistant species. Furthermore, it has been reported (Willemot, 1975) that more PC was synthesized, in wheat seedlings, at low than at high temperatures.

(2) Lima beans at 10°C and 25°C, always had a higher percentage PE than the chilling resistant species (Tables 1 and 2). In addition lima beans had a higher percentage PE at 10°C than at 25°C, while no differences or much smaller differences were observed with change in temperature in the chilling resistant species (Table 3). Combining the results of a higher percentage PE and a lower percentage PC observed in lima beans at 10°C with the fact that PC has a lower Tc than PE, it may be suggested that this chilling sensitive species cannot maintain a proper PC/PE ratio at chilling temperatures. Consequently, its cellular membranes cannot maintain the liquid crystalline phase that is necessary for biological function at low temperatures.

Our results do not agree with the results reported for alfalfa (Kuiper, 1970) where cold resistant varieties exhibited higher PE than cold sensitive ones at low temperature.

(3) A greater percentage PI was observed at 25°C than at 10°C, in most of the cases, suggesting that apparently more PI is synthesized at higher temperature. These results agree with those reported for alfalfa plants (Kuiper, 1970) and rye seedlings (Thomson and Zalik, 1973).

(4) Lima beans exhibit a very large percentage PG at 10°C in both axes and cotyledons compared to the chilling resistant broad beans and peas (Table 3). At 25°C,

there were no large differences between species. This suggests that in the chilling sensitive species (lima beans) there is a shift of metabolism which results in a large amount of PG at the expense, apparently, of PC at 10°C. This large shift in percentage of individual phospholipids do not appear to occur in the chilling resistant species (broad beans and peas). The above results suggest that at chilling temperatures the chilling resistant species are able to maintain a balanced phospholipid metabolism required for their growth and development while chilling sensitive species are not able to do so and consequently are subject to chilling injury. The higher percentage of PG observed at 10°C than at 25°C apparently does not agree with the report on alfalfa plants (Kuiper, 1970) where it was shown that more PG was accumulated at high temperatures than at low ones. The differences observed between axes and cotyledons can be explained, at least partially, by the fact that cotyledons represent an aging tissue while axes represent an actively growing one. Differences in the pattern of phospholipid synthesis between axes and cotyledons have been reported in mung beans (Katayama and Funahashi, 1969) during the incorporation of ^{32}P into lipids.

The possibility that the observed preferential synthesis of some of the phospholipids in cold resistant species or at different temperatures may simply reflect faster turnover of some phospholipids over others at the temperatures and the species studied, cannot be ignored. It should be mentioned that when E. coli cells (Okuyama, 1969) were transferred from 37°C to 10°C, a faster turnover for PE was observed. The phospholipids exhibit various turnover rates in different cellular fractions of the same plant tissue (Mazliak, 1973). The measured "half-lives" in plants varied from 3 to 32 days. Since the incubation period, in this study, was 24 hours and the seeds were analyzed at the end of the incubation period it is assumed that the turnover effect on the data presented here was minimal. The possibility of a differential effect of some temperature treatments on the turnover rates of certain phospholipids could cause changes in phospholipid content without having a cause and effect on cold sensitivity in the plants involved. Furthermore, the possibility of an enzymatic breakdown of the phospholipids during the lipid extraction in this experiment should not be ignored. It has been reported (De La Roche et al., 1973, Quarles and Dawson, 1969), that phospholipase D may cause artifacts during lipid extraction from plant tissues containing water, by breaking phospholipids, and especially PC, into PA and the alcohol moiety of the molecule. By freezing with liquid nitrogen and immediately lyophilizing the plant tissues in this experiment, it is assumed, that the enzymatic breakdown of phospholipids was prevented or at least was minimized to a great degree. In addition, the absence of substantial quantities of PA in the lipid extracts may narrow the possibility of artifacts because of enzymatic breakdown.

Glycerol pool size differences, could contribute to differences observed in labeling of lipids among different species. Pool size differences should be reflected in the total amount of lipids labeled. However, if we assume that the provided labeled glycerol molecules had the same chance to enter phospholipid synthesis as the ones of the cell pool, then, the distribution of the label among the newly synthesized phospholipids (reflected in the percentages of phospholipids discussed here) should not be affected by differences in pool size among the species.

SECTION II

PHOSPHOLIPID SYNTHESIS IN SEEDLINGS OF SOME VEGETABLE SPECIES IN RELATION TO CHILLING RESISTANCE

PHOSPHOLIPID SYNTHESIS IN SEEDLINGS OF SOME VEGETABLE SPECIES IN RELATION TO CHILLING RESISTANCE

Introduction

Chilling sensitive plants are injured by chilling temperatures, 0-10°C (Lyons, 1973), but they can be hardened, at least partially, against chilling injury by conditioning them for relatively short periods of time at temperatures slightly above the chilling temperatures (Wheaton and Morris, 1967; Wilson and Crawford, 1974).

Studies, presented in Section I, indicated marked differences in the pattern of phospholipid synthesis according to chilling sensitivity of the species studied and the temperature during seed germination.

In this study, plants of chilling resistant (broad beans, peas, lettuce, beets and cabbage) and of chilling sensitive (lima beans, cucumber and watermelon) species, in the seedling stage, were employed to gain further information regarding phospholipid synthesis in relation to chilling sensitivity and the development of chilling resistance. The degree of chilling resistance of the species used has been well established (Robinson, 1968; Knott, 1962; Kotowski, 1926).

Materials and Methods

Materials

The seeds used were purchased from Joseph Harris Seed Co., Rochester, N.Y. (beet, cabbage, watermelon, cucumber, lettuce), Northrup, King & Co., Fresno, California (lima bean and pea) and Stokes Seed Co., Buffalo, N.Y. (broad bean).

All the other materials used were the same as in the germination experiments (Section I). The following species and cultivars were used:

1. Beet (*Beta vulgaris*, L., cv. Early Wonder)
2. Broad bean (*Vicia faba*, L., cv. Broad Windsor)
3. Cabbage (*Brassica oleracea*, L., var. capitata cv. Market Price)
4. Cucumber (*Cucumis sativus*, L., cv. Wisconsin SMR 8)
5. Lettuce (*Lactuca sativa*, L., cv. Ruby)
6. Lima bean (*Phaseolus lunatus*, L., cv. Henderson Bush)
7. Peas (*Pisum sativum*, L., cv. Little Marvel)
8. Watermelon [*Citrulus lanatus*, (Thunberg) Matsumura and Nakai, cv. Charleston Gray].

Methods

The seeds were germinated and the seedlings grown in moist vermiculite, in the dark, at 10° (the chilling resistant species) and at 25°C (both chilling resistant and chilling sensitive species) for a designated number of days before they were incubated with ^{14}C-glycerol. Since the chilling sensitive species do not germinate and grow at 10°C there were no treatments of continuous 10°C for these species. For the temperature conditioning (cold hardening) experiment the seedlings were grown for a number of days at 25°C and then transferred to 10°C where they remained for 3 days before they were incubated with ^{14}C-glycerol. The seedlings grown at 10°C and the seedlings of the temperature conditioning treatment were healthy and did not show any chilling injury symptom at the time of incubation with ^{14}C-glycerol.

Within each species the morphological stage of plants treated at 10°C or 25°C or treated after cold hardening was uniform. Each treatment was applied in 3 groups of plants for each species. Details of the characteristics of the treated plants as well as the period of time the several temperature treatments were applied before incubation in glycerol are shown in Table 1. The hypocotyls or epicotyles of plants were cut near the radical and they were soaked for 24 hours in sterilized water containing ^{14}C-glycerol (7.4 mCi/m mole at 0.5 μCi/ml water. Cucumber, especially, was treated with ^{14}C-glycerol of 121 or 133.4 mCi/m mole). At the end of the 24 hour incubation period the plants were frozen in liquid nitrogen and lyophilized to avoid enzymatic degradation of lipids (Hoelzl and

Table 1. Characteristics of seedlings incubated with ^{14}C-glycerol.

Species	25°C			10°C			Temperature Conditioning 25°C→10°C			
	Days at 25°C before treatment	Plant Height cm	Plants per repli-cation	Days at 10°C before treatment	Plant Height cm	Plants per repli-cation	Days at 25°C before transfer to 10°C	Days at 10°C before treatment	Plant Height cm	Plants per repli-cation
Broad Beans	8	12-14	10	--	--	--	5	3	12-14	10
Peas	8	7-8	10	19	7-8	10	5	3	7-8	10
Lettuce	11	5-6	50	21	5-6	50	8	3	5-6	50
Cabbage	11	6-8	50	21	6-8	50	8	3	6-8	50
Beets	11	6-8	50	21	6-8	50	8	3	6-8	50
Lima Beans	6	10-12	10	--	--	--	3	3	10-12	10
Cucumber	11	12-14	12	--	--	--	7	3	12-14	12
Watermelon	8	12-14	12	--	--	--	7	3	12-14	12

Wagner, 1966; Thomson and Zalik, 1973). The lyophylized plants were stored at -30°C until lipid extraction.

The methods for lipid extraction, thin layer chromatography and radioactivity counting were the same as in Section I.

Results

In this study, lipids generally and phospholipids especially were labeled heavily after a 24 hour incubation in ^{14}C-glycerol. The label was found primarily in PA, PI, PC, PE and PG.* Very little label was detected in PS, LPC and Cl, adding up to about 2% of the radioactivity counted in the labeled phospholipids (Appendix, Table A-5). It has been reported (Hölzl and Wagner, 1971; Stobart and Pinfield, 1970) that ^{14}C-glycerol is incorporated, *in vivo*, into several plant phospholipids.

The results are presented as percentages of the CPM recorded for each phospholipid in relation to the total CPM recorded for PI, PC, PE and PG (the major phospholipids in these plants). Phosphatidic acid was not included in the calculations of the percentages of the major phospholipids because PA is considered to be a general intermediate in glyceride synthesis (Van Deenen, 1965; Bishop, 1971;

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*PA: Phosphatidic Acid; PC: Phosphatidyl Choline; PE: Phosphatidyl Ethanolamine; PI: Phosphatidyl Inositol; PG: Phosphatidyl Glycerol; PS: Phosphatidyl Serine; LPC: Lyso-Phosphatidyl Choline; CL: Cardiolipin (Diphosphatidyl Glycerol).

Kates, 1970; Mazliak, 1973; Macher et al., 1975). The results for each phospholipid will be discussed with reference to species and temperature treatments.

The calculations of the percentage of each phospholipid have been made as follows:

$$\text{e.g.:}\quad \frac{\text{CPM for PI}}{\text{CPM for (PI+PC+PE+PG)}} \times 100 = \text{percentage PI}$$

Phosphatidyl Choline (PC)

(1) In the chilling resistant species, with some exceptions, the percentage PC was the highest among the percentages of other phospholipids at 10°C, and was ranked first or second at 25°C and after plants were transferred from 25°C to 10°C (Table 2).

(2) Continuous exposure to 10°C and transfer from 25°C to 10°C generally caused an increased in percentage PC in chilling resistant species compared to the continuous 25°C treatment (Table 3). In chilling sensitive species, transfer from 25°C to 10°C caused an increase in percentage PC compared to the 25°C treatment.

Phosphatidyl Ethanolamine (PE)

(1) In most cases, the percentage PE was the highest or second highest among the percentages of phospholipids in seedlings at 25°C. There was no correlation between percentage PE and the degree of chilling resistance of species (Table 2).

Table 2. Distribution of radioactivity (percentages) among phospholipids of seedlings after a 24 hour incubation with ^{14}C-glycerol at 10°C or 25°C. Comparison of individual phospholipids.

Species	Tempera-ture °C	Phospholipid Percentages				L.S.D. (0.05)
		PI	PC	PE	PG	
Broad Beans	10°C	--	--	--	--	--
	25→10°C	13.37	58.46	10.30	17.84	1.25
	25°C	14.49	52.81	21.73	10.93	2.57
Peas	10°C	20.63	40.40	17.40	21.43	5.41
	25→10°C	21.22	45.07	12.83	20.86	3.04
	25°C	17.11	43.98	23.75	13.79	1.74
Lettuce	10°C	12.66	39.09	26.19	22.03	3.38
	25→10°C	10.48	45.30	24.20	19.98	1.86
	25°C	23.81	18.84	42.85	14.47	7.28
Beets	10°C	10.87	23.42	41.59	24.08	5.79
	25→10°C	14.58	32.13	31.02	22.24	2.78
	25°C	13.21	30.65	39.60	16.50	4.13
Cabbage	10°C	10.65	34.80	29.15	25.36	2.30
	25→10°C	17.09	32.19	27.83	22.93	3.02
	25°C	10.82	28.84	39.27	21.04	5.57
Lima Beans	10°C	--	--	--	--	--
	25→10°C	24.01	49.71	14.40	11.85	2.36
	25°C	21.80	38.07	25.62	14.47	2.66
Cucumber	10°C	--	--	--	--	--
	25→10°C	22.27	42.48	19.57	15.65	1.75
	25°C	6.37	29.13	53.70	10.77	2.69
Watermelon	10°C	--	--	--	--	--
	25→10°C	20.92	40.27	24.61	14.18	4.79
	25°C	20.51	33.82	34.69	10.96	3.40

Table 3. Distribution of radioactivity (percenrages) among phospholipids of seedlings after a 24 hour incubation with ^{14}C-glycerol at 10°C or 25°C. Comparison of temperatures and species.

Phospho-lipid	Tempera-ture °C	Chilling resistant					Chilling sensitive			L.S.D. (0.05)
		Broad Beans	Peas	Lettuce	Beets	Cabbage	Lima Beans	Cucumber	Watermelon	
PC	10	--	40.40	39.09	23.42	34.80	--	--	--	10.24
PC	25→10	58.46	45.07	45.30	32.13	32.19	49.71	42.48	40.27	3.25
PC	25	52.81	43.98	18.84	30.65	28.84	38.07	29.13	33.82	4.18
L.S.D. (0.05)		3.48	N.S.	5.33	5.46	4.00	3.05	3.37	6.41	
PE	10	--	17.40	26.19	41.59	29.15	--	--	--	5.17
PE	25→10	10.30	12.83	24.20	31.02	27.83	14.40	19.57	24.61	2.39
PE	25	21.73	23.75	42.85	39.60	39.27	25.62	53.70	34.69	4.87
L.S.D. (0.05)		1.74	2.04	7.28	6.56	4.56	3.81	3.28	4.24	
PI	10	--	20.63	12.66	10.87	10.65	--	--	--	4.20
PI	25→10	13.37	21.22	10.48	14.58	17.09	23.68	22.56	20.51	1.88
PI	25	14.49	17.11	23.81	13.21	10.82	21.80	6.37	20.51	3.04
L.S.D. (0.05)		N.S.	N.S.	2.93	N.S.	3.51	N.S.	1.80	N.S.	
PG	10	--	21.43	22.03	24.08	25.36	--	--	--	2.08
PG	25→10	17.84	20.86	19.98	22.24	22.93	11.85	15.65	14.18	2.51
PG	25	10.93	13.79	14.47	16.50	21.04	14.47	10.77	10.96	2.68
L.S.D. (0.05)		1.93	2.39	3.44	1.69	N.S.	0.58	2.16	N.S.	

(2) Generally, a higher percentage PE was observed at 25°C than at 10°C in all chilling resistant species, except beets. Chilling sensitive species exhibited much lower percentage PE when transferred from 25°C to 10°C than at 25°C continuously (Table 3).

Phosphatidyl Inositol (PI)

(1) Although significant differences observed in percentage PI between species at 25°C and when transferred from 25°C to 10°C, no consistent differences occurred between chilling sensitive and chilling resistant species (Table 3).

(2) Among the temperature conditioned plants the percentage PI was generally higher in chilling sensitive species with the exception of peas (Table 3).

Phosphatidyl-glycerol (PG)

(1) A higher percentage PG was observed in chilling resistant species at 10°C than at 25°C with the exception of cabbage (Table 3).

(2) The temperature conditioning treatment (transfer from 25°C to 10°C) resulted in an increase of percentage PG in comparison to 25°C in most of the species (Table 3). Among the chilling hardened plants, most chilling resistant species had higher percentage PG than the chilling sensitive ones (Table 3) and PG was a higher ranking phospholipid in chilling resistant species (Table 2).

Phosphatidyl Choline / Phosphatidyl Ethanolamine Ratio

(1) In general, a higher PC/PE ratio was found at 10°C than at 25°C in the chilling resistant species (Table 4).

(2) The temperature conditioning treatment (25°C→ 10°C) resulted in an increase of the PC/PE ratio in comparison to the one at 25°C, in all chilling resistant and sensitive species (Table 4).

Phospholipids as Percent of Total Lipids*

It has been reported that during the development of cold hardiness (Siminovitch et al., 1968; Yoshida and Sakai, 1973) or growth at low temperatures (Kuiper, 1970; De La Roche et al., 1972), some plant species accumulate increasing quantities of phospholipids. The radioactivity incorporated in all phospholipids in relation to the radioactivity incorporated in all lipids was calculated with the following results:

(1) In chilling resistant species no significant or consistent differences were observed between 10°C and 25°C in total quantities of phospholipids synthesized in relation to total lipids (Table 4).

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*PA was not included in the calculations.

Table 4. Comparison of radioactivity distributed (percentages) in phosphatidyl choline vs. phosphatidyl ethanolamine, in total phospholipids vs. total lipids, and in phosphatidic acid vs. total lipids, after a 24 hour incubation with ^{14}C-glycerol at 10°C or 25°C.

Phospholipid ratio	Temperature °C	Chilling resistant					Chilling Sensitive			L.S.D. (0.05)
		Broad Beans	Peas	Lettuce	Beets	Cabbage	Lima Beans	Cucumber	Watermelon	
$\frac{PC}{PE}$	10	--	2.331	1.493	0.573	1.197	--	--	--	0.378
"	25→10	5.686	3.526	1.870	1.035	1.161	3.483	2.171	1.654	0.485
"	25	2.431	1.853	0.454	0.779	0.732	1.491	0.543	0.975	0.175
L.S.D. (0.05)		0.698	0.601	0.232	0.022	0.013	0.728	0.163	0.474	
$\frac{\text{Phospholipid} \times 100}{\text{Total lipid}}$	10	--	41.01	53.46	44.36	53.80	--	--	--	5.41
"	25→10	61.85	47.76	54.12	42.90	37.91	43.56	64.42	32.24	6.26
"	25	53.30	58.50	28.04	44.66	62.60	46.94	76.37	33.81	8.54
L.S.D. (0.05)		6.91	4.00	7.71	N.S.	13.32	N.S.	7.67	N.S.	
$\frac{PA \times 100}{\text{Total Lipid}}$	10	--	31.13	15.15	26.98	16.57	--	--	--	4.86
"	25→10	14.41	26.71	11.70	20.93	24.20	18.31	8.58	37.21	5.87
"	25	14.37	24.43	39.60	27.49	21.88	19.36	0.98	37.70	7.76
L.S.D. (0.05)		N.S.	3.58	5.27	N.S.	N.S.	N.S.	0.63	N.S.	

(2) The degree of chilling resistance in species was not correlated to the total phospholipids synthesized (Table 4).

(3) In most of the treated species the chilling hardening caused few alterations in the total amount of phospholipids synthesized in comparison to the 25°C treatment.

Phosphatidic Acid as Percent of Total Phospholipid

The phosphatidic acid labeled in comparison to the labeled total lipids showed no consistent relationships to the degree of chilling resistance of the species, the temperature of growth or the temperature conditioning treatment (Table 4).

Discussion

The *de novo* synthesis of phospholipids is believed to be essential for the building of new cell and subcellular membranes as well as for the maintenance of the existing membranes (Van Deenen, 1965).

In our labeling studies, with a variety of chilling sensitive and chilling resistant vegetable species, lipids and especially phospholipids were labeled heavily over an incubation period of 24 hours.

It has been reported (Chapman, 1967; Williams and Chapman, 1970) that phospholipids, natural or artificial, c

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change phase from a liquid crystalline to a crystalline state at temperatures much lower than their melting points. This change may be of importance since phospholipids are major components of membranes. According to those studies PC changes phase at a much lower temperature than PE (Appendix, Table A-3). This difference could be important in relation to the function of membranes of chilling resistant and chilling sensitive species at low temperatures if the membranes contain differing amounts or ratios of PC and PE.

More PC has been found at low temperatures in cold resistant alfalfa cultivars than in cold sensitive ones (Kuiper, 1970) as well as in wheat seedlings grown at low rather than high temperatures (Willemot, 1975).

The results of this study indicate that exposure of chilling resistant plants to 10°C continuously, or shifting chilling resistant and sensitive plants from 25°C to 10°C generally causes an increase in the percentage PC in comparison with the continuous 25°C treatment (Table 3). A similar difference in percentage PG was also noted when plants of chilling resistant species grown at 10°C or transferred from 25°C to 10°C compared to plants grown continuously at 25°C (Table 3).

These results suggest that an increase in PC and possibly PG upon exposure to low temperatures could be contributing to hardening of both chilling resistant and sensitive species. Presumably, the higher PC and PG percentages would allow the membranes to maintain a liquid-crystalline phase at 10°C and normal functioning could occur. In contrast, the percentage PE in chilling resistant species was lower when the seedlings were grown at 10°C than at 25°C. In addition, conditioning plants, by transferring them from 25°C to 10°C, resulted in a decrease in percentage PE in both chilling resistant and sensitive species compared to those plants grown at 25°C continuously.

PI was found to be a higher ranking phospholipid in chilling sensitive species than in chilling resistant ones when temperature conditioned plants were compared (Table 2). Chilling sensitive species had a higher percentage of PI than the majority of chilling resistant ones under lower temperature conditions (Table 3). PI may play some role in chilling sensitivity.

The percentage PE appears to decrease and the percentage PC increases when chilling sensitive and chilling resistant plants are transferred from 25°C to 10°C. This suggests that the low temperature causes a shift in metabolism of phospholipids towards PC at the expense of PE. This may contribute to the synthesis of membranes which will remain flexible and will function at low temperature.

This shift is reflected in the PC/PE ratio comparing seedlings exposed to 25°C or grown at 25°C and transferred to 10°C. Exposure to low temperature results in an increase in the PC/PE ratio.

There appears to be a basic difference in how chilling sensitive lima beans respond to low temperature treatment (in terms of phospholipid synthesis) between the initial imbibition stage of germination and the seedling stage. It was shown earlier (Section I) that lima beans synthesized less PC and more PE at 10°C than at 25°C during the seed imbibition stage. Large changes in PC and PE synthesis did not occur in broad beans or peas when imbibed at 10°C or 25°C.

In addition, lima beans exhibited a significantly lower percentage PC and a higher percentage PE at 10°C than broad beans and peas. It is known that lima beans are extremely sensitive to chilling injury during this early imbibition stage (Pollock and Toole, 1966; Woodstock and Pollock, 1965).

In the seedling stage both the chilling resistant species (broad beans and peas) and the chilling sensitive one (lima beans) synthesized more PC and less PE after they were transferred from 25°C to 10°C than at 25°C. Lima beans are less sensitive to cold temperatures after the initial stages of imbibition have passed (Pollock and Toole, 1966).

This information suggests that lima beans in the imbibition stage are not capable of shifting metabolism to produce the type of phospholipids necessary at low temperature for proper maintenance of cellular integrity, and chilling injury and death of the seed occurs. Lima beans (as well as cucumber and watermelon) in the seedling stage appear to have the capability, at low temperatures, of shifting metabolism to higher PC and lower PE levels. This is similar to the chilling resistant species. Some degree of hardening apparently occurs that protects the chilling sensitive plant from chilling injury, as occurs in the cold resistant species. In this experiment, the temperature conditioned chilling sensitive plants were observed to stay alive, but with a small growth rate, for about 30 days at 10°C. This may indicate that, in addition to a proper phospholipid metabolism, some other factors determine growth at chilling temperatures.

However the possibility that a 24 hour phospholipid synthesis may not represent the phospholipid synthesis mechanism in the studied plants at different temperatures should not be ignored and therefore any conclusions should be made cautiously.

The possibility also exists that our data have been affected by a differential effect of temperature on the turnover rates of the several phospholipid classes resulting in a picture that may have nothing to do with the mechanism of chilling resistance (see discussion in Section I). Furthermore, there is a possibility that an artifact, because of enzymatic degradation of lipids during extraction, altered the constituents of the analyzed lipid mixture. In fact it has been reported (De La Roche et al., 1973; Quarles and Dawson, 1969) that phospholipase D can cause artifacts during lipid extraction, in the presence of water, by breaking phospholipids, especially PC, into PA and the alcoholic moiety of the molecule.

It is assumed that the possibility of an enzymatic degradation of phospholipids during the procedure of lipid extraction from the tissues was minimized by the freezing in liquid nitrogen and lyophilization of the plants (Hoelzl and Wagner, 1966; Thomson and Zalik, 1973).

The fact that PA was heavily labeled in the extracted lipids (Table 4) may support the idea of an artifact because of an enzymatic breakdown of phospholipids by phospholipase D. But since PA is a precursor in glyceride synthesis (Kates, 1970; Mazliak, 1973; Macher _et al_., 1975) we suggest that the labeled PA was synthesized _de novo_ from ^{14}C-glycerol as an intermediate step for lipid synthesis. A high PA concentration has been reported in other cases (De La Roche _et al_., 1973; Wilson and Rinne, 1974) and it was explained as synthesized _de novo_.

The comparison of the pattern of lipid synthesis of different species may be subject to an error because of differences in pool size of glycerol as it has been discussed earlier (Section I).

SECTION III

FATTY ACID CONTENT OF PHOSPHOLIPIDS IN GERMINATING SEEDS OF SOME CHILLING RESISTANT AND CHILLING SENSITIVE VEGETABLE SPECIES

FATTY ACID CONTENT OF PHOSPHOLIPIDS IN GERMINATING SEEDS OF SOME CHILLING RESISTANT AND CHILLING SENSITIVE VEGETABLE SPECIES

Introduction

Chilling injury during the early stages of seed imbibition in chilling sensitive species has been shown to cause failure of germination or abnormal seedlings (Obendorf and Hobbs, 1970; Pollock and Toole, 1966; Pollock, 1969; Woodstock and Pollock, 1965; Christianson, 1968). Chilling sensitivity and chilling injury have been correlated to a phase change occurring in lipids of cellular membranes of chilling sensitive plants at chilling temperatures, 0-12°C, (Lyons and Raison, 1970; Raison *et al*., 1971b; Shneyour *et al*., 1973; Towers *et al*., 1972; Towers *et al*., 1973; Yamaki and Uritani, 1974; Phillips and McWilliam, 1971). Such a phase change of lipids occurs at much lower temperatures in chilling resistant plants.

Membrane lipids of chilling sensitive plants have been found to contain less unsaturated fatty acids than those of chilling resistant plants (Lyons *et al*., 1964). In addition, membrane lipids of micro-organisms and plants grown at high temperatures contain less unsaturated fatty acids than those from organisms grown at low temperatures (Haest et al., 1969; Miller et al., 1974).

The higher the unsaturation of a fatty acid the lower its melting point (Appendix, Table A-2). It has been suggested that the correct fluidity of biological membranes, for function at a particular temperature, is provided by the distribution in the membrane lipids of fatty acids with the proper chain length and degree of unsaturation (Chapman, 1967 and 1968; Cronan and Vagelos, 1972).

Phospholipids, which are important cell membrane components (Bishop, 1971; Chapman, 1967; Van Deenen, 1965) have been found to be more unsaturated when growth occurs at low temperatures, in some micro-organisms (Kaneda, 1972; Cronan and Vagelos, 1972; Kates and Paradis, 1973) alfalfa (Grenier and Willemot, 1974) and wheat plants (De La Roche et al., 1972). Cold resistant alfalfa varieties also had more unsaturated phospholipids than did sensitive ones (Grenier and Willemot, 1974).

Since cellular lipids apparently are involved in the cold sensitivity of plants, knowledge of the distribution of the fatty acids in phospholipids of germinating seeds of chilling sensitive and chilling resistant plant species may contribute to an understanding of the chilling injury mechanism. Chilling sensitive lima beans and chilling resistant broad beans and peas were employed to investigate the relationship of phospholipid fatty acid composition to chilling sensitivity.

Materials and Methods

Materials

Seeds and other materials described earlier (Section I) were also used for the study of fatty acids in phospholipids.

Methods

Seeds of lima beans, broad beans and peas were surface sterilized with 1 percent NaOCl and imbibed for 24 hours in distilled water at 10°C or 25°C in the dark. At the end of the imbibition period the seeds were frozen in liquid nitrogen and lyophilized before lipid extraction. Other groups of seeds were maintained at 10°C, in moist vermiculite in the dark, for 6 days before they were analyzed for fatty acid content of phospholipids.

Methods for lipid extraction and separation of phospholipids by thin layer chromatography were described earlier (Section I).

The phospholipids were located on the thin layer plates by spraying with 2',7'-dichloro fluorescein and observation under UV light (Mackender and Leech, 1974).

Fatty Acid Analysis. The fatty acids of phosphatidyl inositol (PI) phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC) were determined.

The phospholipid areas were scraped from the thin layer plates and the lipids were eluted from the silica gel using chloroform: methanol: water (10:10:0.5 v/v/v). The methyl esters of the fatty acids of the phospholipids were prepared using BF_3-methanol according to Metcalfe et al., 1966; after saponification of the phosphalipids with 0.5N NaOH in methanol the free fatty acids were methylated with BF_3-methanol (14% w/v). The methyl esters of the fatty acids were removed from the reaction medium by partitioning in hexane for analysis by gas liquid chromatography (GLC).

GLC Analysis. The fatty acid composition of the methyl esters was determined using a Packard 7300 gas-liquid chromatograph equipped with a flame ionization detector. A 1.80M, 3mm I.D. glass column packed with 10% SP-222 PS (brand name of Supelco, Inc. for diethylene glycol succinate) was employed at a temperature of 190°C. The temperature of the injector and the detector was 220°C and the flow rate of the nitrogen carrier gas was 50 ml/min.

Authentic standards were used for the identification of the methyl ester peaks in the chromatograms. The major fatty acids (representing more than 90% of the total fatty acids) were identified as 16:0, 18:0, 18:1, 18:2

and 18:3* (the 1st number indicating the number of carbon atoms and the second one the number of double bonds in the molecule).

The relative concentration of each fatty acid was calculated by triangulation of the peak areas on the chromatograms (corrections were made for detector sensitivity) and was expressed as percentage of the total peak area

$$\text{e.g.: } \frac{16{:}0}{16{:}0+18{:}0+18{:}1+18{:}2+18{:}3} \times 100 = \text{percentage } 16{:}0$$

Other calculations made were:

$$(1) \quad \frac{\text{Unsaturated}}{\text{Saturated}} \text{ ratio} = \frac{18{:}1+18{:}2+18{:}3}{16{:}0+18{:}0}$$

$$(2) \quad \begin{array}{l}\text{Double Bond}\\ \text{Index (D.B.I.)}\end{array} = \frac{(\%\text{ of }18{:}1)\text{x}1+(\%\text{ of }18{:}2)\text{x}2+(\%\text{ of }18{:}3)\text{x}3}{100}$$

Results

Content of Fatty Acids in Phosphatidyl Choline

(1) Species Differences. Considerable differences in percentage of individual fatty acids were observed between species during both imbibition and after 6 days of germination.

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*16:0: Palmitic; 18:0: Stearic; 18:1: Oleic; 18:2: Linoleic; 18:3: Linolenic.

(a) Lima beans exhibited a considerably higher percentage 16:0 in both axes and cotyledons during the imbibition stage (Table 1) and in cotyledons on the 6th day of germination (Table 2) than did the chilling resistant species.

(b) The percentage 18:1 was much higher in peas and broad beans (cotyledons and axes) than in lima beans during imbibition (Table 1). However the percentage 18:1 on the 6th day was different from the imbibition stage. On the 6th day the percentage 18:1 in broad bean and pea axes was much lower than in lima beans while the cotyledons of peas and broad beans had the highest percentage 18:1 (Table 3).

(c) The situation for 18:2 of cotyledons and axes was mixed, with lima beans showing a higher percentage in cotyledons during imbibition (Table 1) and a lower percentage in axes on the 6th day (Table 3). No other consistent relationship occurred.

(d) Lima beans, in all cases, had a much higher percentage 18:3 than the chilling resistant species (Tables 1 and 3).

(e) The double bond index (D.B.I.) of lima beans was larger than the chilling resistant species during imbibition (Table 3) at 10°C and 25°C. However, on the 6th day, in cotyledons, lima beans had the lowest D.B.I. (Table 3).

Table 1. Phosphatidyl choline: Percentages of fatty acids after 24 hours of germination at 10°C or 25°C. Comparison of species.

Temp.	Species	Seed part	Fatty Acids 16:0	18:0	18:1	18:2	18:3	Double Bond Index	Unsat./Sat.
10°C	Broad	Axes	9.84	1.86	32.66	52.47	3.13	1.47	7.57
10°C	Peas	Axes	9.70	1.83	44.84	38.00	5.60	1.37	7.68
10°C	Lima Beans	Axes	15.48	3.58	18.45	42.54	19.91	1.63	4.30
L.S.D.(0.05)			2.94	0.94	4.37	4.23	2.77	0.07	1.19
25°C	Broad Beans	Axes	11.52	4.15	35.47	45.81	3.02	1.36	5.44
25°C	Peas	Axes	8.95	3.86	45.97	35.75	5.42	1.33	6.80
25°C	Lima Beans	Axes	20.38	4.24	19.68	36.21	19.44	1.50	3.13
L.S.D.(0.05)			5.20	N.S.	2.63	5.05	1.61	0.10	1.32
10°C	Broad Beans	Cotyl.	7.93	1.90	48.03	40.30	1.90	1.34	9.22
10°C	Peas	Cotyl.	10.63	5.04	35.59	44.38	4.31	1.37	5.42
10°C	Lima Beans	Cotyl.	20.06	3.72	8.74	54.99	12.46	1.56	3.21
L.S.D.(0.05)			2.10	1.77	4.69	5.34	2.42	0.05	1.21
25°C	Broad Beans	Cotyl.	12.08	2.68	48.22	35.37	1.61	1.24	5.86
25°C	Peas	Cotyl.	10.65	4.81	36.79	42.99	4.71	1.36	5.47
25°C	Lima Beans	Cotyl.	21.49	2.82	8.90	54.09	12.65	1.55	3.14
L.S.D.(0.05)			4.11	1.54	6.37	5.60	1.47	0.09	1.31

Table 2. Phosphatidyl choline: Percentages of fatty acids after 24 hours of germination at 10°C or 25°C. Comparison of temperatures and seed parts.

Fatty Acids	Seed part	Broad Beans 10°C	Broad Beans 25°C	Broad Beans L.S.D. (0.05)	Peas 10°C	Peas 25°C	Peas L.S.D. (0.05)	Lima Beans 10°C	Lima Beans 25°C	Lima Beans L.S.D. (0.05)
16:0	Axes	9.84	11.52	N.S.	9.70	8.96	N.S.	15.48	20.38	N.S.
	Cotyl.	7.93	12.08	4.16	10.63	10.65	N.S.	20.06	21.49	N.S.
L.S.D.(0.05)		1.52	N.S.		N.S.	1.67		4.61	N.S.	
18:0	Axes	1.86	4.15	1.12	1.83	3.86	1.04	3.58	4.24	N.S.
	Cotyl.	1.90	2.68	N.S.	5.04	4.81	N.S.	3.72	2.82	N.S.
L.S.D.(0.05)		N.S.	N.S.		2.37	N.S.		N.S.	1.41	
18:1	Axes	32.66	35.47	2.32	44.84	45.97	N.S.	18.45	19.68	N.S.
	Cotyl.	48.03	48.22	N.S.	35.59	36.79	N.S.	8.74	8.90	N.S.
L.S.D.(0.05)		5.38	6.26		5.96	6.79		3.86	2.41	
18:2	Axes	52.47	45.80	5.37	38.00	35.75	N.S.	42.54	36.21	4.89
	Cotyl.	40.30	35.37	N.S.	44.38	43.12	N.S.	54.99	54.07	N.S.
L.S.D.(0.05)		4.32	6.53		5.38	5.26		6.48	5.68	
18:3	Axes	3.13	3.02	N.S.	5.60	5.42	N.S.	19.91	19.44	N.S.
	Cotyl.	1.90	1.61	N.S.	4.31	4.71	N.S.	12.46	12.65	N.S.
L.S.D.(0.05)		0.363	0.57		N.S.	N.S.		4.85	2.86	
Unsat./Sat.	Axes	7.57	5.44	1.70	7.65	6.80	0.81	4.30	3.13	N.S.
	Cotyl.	9.22	5.86	2.02	5.42	5.47	N.S.	3.21	3.14	N.S.
L.S.D.(0.05)		1.64	N.S.		1.18	0.82		N.S.	N.S.	
Double Bond Index	Axes	1.47	1.36	0.08	1.37	1.33	N.S.	1.63	1.50	N.S.
	Cotyl.	1.34	1.24	0.05	1.37	1.36	N.S.	1.56	1.55	N.S.
L.S.D.(0.05)		0.05	0.09		N.S.	N.S.		N.S.	N.S.	

Table 3. Percentages of fatty acids in phospholipids (PC, PE, PI) after 6 days of germination at 10°C. Comparison of species.

Phospholipid	Species	Seed part	Fatty Acids 16:0	18:0	18:1	18:2	18:3	Double Bond Index	Unsat./Sat.
PC	Broad Beans	Axes	11.60	1.81	3.53	71.01	12.00	1.81	6.67
PC	Peas	Axes	16.48	3.04	4.93	61.48	14.04	1.70	4.12
PC	Lima Beans	Axes	12.69	3.17	19.13	43.41	21.54	1.70	5.42
	L.S.D.(0.05)		2.83	N.S.	1.38	4.84	5.30	0.09	1.60
PC	Broad Beans	Cotyl.	9.79	1.82	33.11	52.91	2.34	2.92	7.72
PC	Peas	Cotyl.	7.78	5.29	22.65	58.93	5.30	3.03	6.70
PC	Lima Beans	Cotyl.	22.57	3.20	7.26	53.32	10.02	1.44	2.81
	L.S.D.(0.05)		3.85	1.22	6.81	N.S.	1.15	0.294	1.85
PE	Broad Beans	Axes	18.59	2.92	2.98	64.84	13.15	1.72	3.99
PE	Peas	Axes	22.43	3.07	3.33	55.74	15.16	1.61	2.97
PE	Lima Beans	Axes	25.12	2.48	15.81	40.53	16.04	1.45	2.63
	L.S.D.(0.05)		N.S.	N.S.	1.32	13.61	N.S.	N.S.	N.S.
PE	Broad Beans	Cotyl.	13.59	2.71	29.59	51.75	2.31	1.40	5.20
PE	Peas	Cotyl.	15.35	5.64	16.54	58.45	3.97	1.45	3.79
PE	Lima Beans	Cotyl.	27.90	2.23	5.78	55.22	8.83	1.42	2.34
	L.S.D.(0.05)		4.93	0.99	5.87	N.S.	1.23	N.S.	1.17
PI	Broad Beans	Axes	37.68	3.45	2.93	46.69	9.20	1.24	1.44
PI	Peas	Axes	32.13	4.24	4.29	42.40	16.95	1.40	1.75
PI	Lima Beans	Axes	30.18	5.55	10.75	31.12	22.37	1.40	1.80
	L.S.D.(0.05)		N.S.	0.80	1.23	3.64	3.22	0.10	N.S.
PI	Broad Beans	Cotyl.	32.95	3.57	16.81	45.27	1.36	1.11	1.75
PI	Peas	Cotyl.	34.74	7.81	12.79	39.81	4.81	1.07	1.35
PI	Lima Beans	Cotyl.	36.31	5.86	4.91	42.13	10.74	1.21	1.42
	L.S.D.(0.05)		N.S.	1.36	3.13	N.S.	1.56	N.S.	N.S.

(f) During imbibition in both axes and cotyledons, and in cotyledons only on the 6th day, peas and broad beans had a higher ratio of unsaturated to saturated fatty acids than lima beans (Tables 1 and 3).

(2) Cotyledons vs. Axes. When cotyledons and axes were compared there were differences in percentage of individual fatty acids in the imbibition stage but no consistent trends were evident in both chilling resistant and sensitive species (Table 2). For example, the percentage 18:1 was higher in cotyledons than axes of broad beans and lima beans while the opposite was true in peas.

After 6 days of germination at 10°C (Table 4) the following were observed:

(a) The cotyledons of the chilling resistant species had a much higher percentage 18:1 than the axes while the opposite was true in lima beans.

(b) The percentage 18:3 was higher in the axes than in cotyledons, in all species.

(c) The D.B.I. was larger in cotyledons in comparison to axes of the chilling resistant plants but in lima beans the opposite was true.

(d) The cotyledons of chilling resistant species had the same or higher unsaturated/saturated ratio than the corresponding axes while the axis tissue of lima beans had a higher ratio than the cotyledonary tissue.

Table 4. Percentages of fatty acids in phospholipids (PC, PE, PI) after 6 days of germination at 10°C. Comparison of seed parts.

Phospho-lipid	Species	Seed Part	Fatty Acids 16:0	18:0	18:1	18:2	18:3	Double Bond Index	Unsat./Sat.
PC	Broad Beans	Axes	11.60	1.81	3.53	71.01	12.00	1.81	6.66
		Cotyl.	9.79	1.82	33.11	52.91	2.34	2.92	7.72
	L.S.D.(0.05)		N.S.	N.S.	7.94	9.09	2.22	0.257	N.S.
PC	Peas	Axes	16.48	3.04	4.93	61.48	14.04	1.70	4.12
		Cotyl.	7.78	5.29	22.65	58.93	5.30	3.03	6.70
	L.S.D.(0.05)		3.08	1.650	5.37	N.S.	6.638	0.247	1.40
PC	Lima Beans	Axes	12.69	3.17	19.13	43.41	21.54	1.70	5.42
		Cotyl.	22.57	3.20	7.26	53.32	10.02	1.44	2.81
	L.S.D.(0.05)		5.55	N.S.	1.19	N.S.	2.79	0.240	2.06
PE	Broad Beans	Axes	18.59	2.92	2.98	64.84	13.15	1.72	3.99
		Cotyl.	13.59	2.71	29.59	51.75	2.31	1.40	5.20
	L.S.D.(0.05)		N.S.	N.S.	5.99	N.S.	1.41	N.S.	N.S.
PE	Peas	Axes	22.43	3.07	3.33	55.74	15.16	1.61	2.97
		Cotyl.	15.35	5.64	16.54	58.45	3.97	1.45	3.79
	L.S.D.(0.05)		5.69	1.46	5.05	N.S.	4.29	0.08	N.S.
PE	Lima Beans	Axes	25.12	2.48	15.81	40.53	16.04	1.45	2.63
		Cotyl.	27.90	2.43	5.78	55.22	8.83	1.42	2.34
	L.S.D.(0.05)		N.S.	N.S.	2.88	8.74	1.12	N.S.	N.S.
PI	Broad Beans	Axes	37.68	3.45	2.93	46.69	9.20	1.24	1.44
		Cotyl.	32.50	3.57	16.81	45.27	1.36	1.11	1.74
	L.S.D.(0.05)		N.S.	N.S.	4.36	N.S.	1.27	0.10	N.S.
PI	Peas	Axes	32.13	4.24	4.29	42.40	16.95	1.40	1.75
		Cotyl.	34.74	7.81	12.79	39.81	4.81	1.07	1.35
	L.S.D.(0.05)		2.52	1.00	1.37	N.S.	2.39	0.04	0.148
PI	Lima Beans	Axes	30.18	5.55	10.75	31.12	22.37	1.40	1.80
		Cotyl.	36.31	5.86	4.91	42.13	10.74	1.21	1.42
	L.S.D.(0.05)		N.S.	N.S.	0.97	N.S.	4.17	N.S.	N.S.

(3) 25°C vs. 10°C Imbibition. When imbibition at 10°C was compared with imbibition at 25°C no consistent differences in the percentage of any fatty acids could be detected in any of the species and no relationship was seen between chilling resistant and sensitive species.

(4) Imbibition Stage vs. 6th Day of Germination at 10°C. There was a large change in percentage of individual fatty acids between imbibition and the 6th day of germination at 10°C in broad beans and peas but not in lima beans (Table 5), presumably because lima beans cannot germinate at that temperature and consequently they do not have an active lipid metabolism.

Specifically, in axis tissue of broad beans and peas, there was an increase in percentage 16:0, 18:2 and 18:3 and a decrease in percentage 18:1 when comparing fatty acid content at the initial imbibition stage and after 6 days germination of 10°C (Table 5).

In cotyledons, there was an increase in percentage 18:2 and a decrease in percentage 18:1 the 6th day of germination compared to the imbibition stage in broad beans and peas (Table 5). There was little or no change in lima beans.

Table 5. Phosphatidyl choline: Percentages of fatty acids after 24 hours or 6 days of germination at 10°C.

Species	Seed part	Germination stage	Fatty Acids 16:0	18:0	18:1	18:2	18:3	Double Bond Index	Unsat./Sat.
Broad Beans	Axes	24th hr.	9.84	1.86	32.56	52.47	3.13	1.47	7.57
	Axes	6th day	11.60	1.81	3.53	71.01	12.00	1.81	6.67
L.S.D.(0.05)			1.68	N.S.	1.45	3.33	1.89	0.05	N.S.
Peas	Axes	24th hr.	9.70	1.83	44.84	38.00	5.60	1.37	7.68
	Axes	6th day	16.48	3.04	4.93	61.48	14.04	1.70	4.12
L.S.D.(0.05)			1.83	N.S.	4.70	6.95	6.63	0.10	0.54
Lima Beans	Axes	24th hr.	15.48	3.58	18.45	42.54	19.91	1.63	4.30
	Axes	6th day	12.69	3.17	19.13	43.41	21.54	1.70	5.42
L.S.D.(0.05)			N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Broad Beans	Cotyl.	24th hr.	7.93	1.90	48.03	40.30	1.90	1.34	9.22
	Cotyl.	6th day	9.79	1.82	33.11	52.91	2.34	2.92	7.72
L.S.D.(0.05)			1.78	N.S.	9.46	9.50	N.S.	0.25	N.S.
Peas	Cotyl.	24th hr.	10.63	5.04	35.59	44.38	4.31	1.37	5.42
	Cotyl.	6th day	7.78	5.29	22.65	58.93	5.30	3.03	6.70
L.S.D.(0.05)			2.80	N.S.	6.50	4.82	N.S.	0.23	N.S.
Lima Beans	Cotyl.	24th hr.	20.06	3.72	8.74	54.99	12.46	1.56	3.21
	Cotyl.	6th day	22.57	3.20	7.25	53.32	10.02	1.44	2.81
L.S.D.(0.05)			N.S.	N.S.	0.62	N.S.	N.S.	N.S.	N.S.

Content of Fatty Acids in Phosphatidyl Ethanolamine

(1) Species Differences.

(a) Lima beans had a considerably higher percentage 16:0 than the chilling resistant species, except in axis tissue at 10°C, both during imbibition (Table 6) and the 6th day of germination at 10°C (Table 3).

(b) The chilling resistant species had a higher percentage 18:1 than lima beans in most cases (except axis of lima beans on 6th day) (Tables 6 and 3).

(c) In general, the percentage 18:3 was highest in lima beans.

(d) In most cases, the unsaturated/saturated ratio was highest for the chilling resistant species and lowest for lima beans (Tables 6 and 3).

(e) The D.B.I. was not related to chilling resistance or sensitivity.

(2) Axes vs. Cotyledons. During imbibition, the main difference between axes and cotyledons was that broad beans and peas had the same or higher percentage 18:1 in cotyledons than in axis tissue while lima beans had a considerably higher percentage 18:1 in axis tissue (Table 7).

On the 6th day, broad beans and peas had a higher percentage 18:1 in cotyledons than in the axes. Lima beans

Table 6. Phosphatidyl ethanolamine: Percentages of fatty acids after 24 hours of germination at 10°C or 25°C. Comparison of species.

Temp.	Species	Seed part	Fatty Acids 16:0	18:0	18:1	18:2	18:3	Double Bond Index	Unsat./Sat.
10°C	Broad Beans	Axes	12.37	2.34	27.64	54.90	2.77	1.45	6.06
10°C	Peas	Axes	18.03	5.33	36.20	37.23	3.17	1.20	3.35
10°C	Lima Beans	Axes	19.17	2.97	16.52	45.82	14.14	1.50	3.50
L.S.D.(0.05)			N.S.	N.S.	3.64	5.96	2.35	0.136	2.13
25°C	Broad Beans	Axes	13.33	3.88	22.39	58.33	2.04	1.45	4.90
25°C	Peas	Axes	10.53	3.71	38.06	43.07	4.59	1.38	6.13
25°C	Lima Beans	Axes	38.94	2.52	12.61	28.79	17.11	1.22	1.42
L.S.D.(0.05)			5.48	N.S.	7.33	12.18	3.97	0.141	1.66
10°C	Broad Beans	Cotyl.	12.30	2.05	36.00	47.96	1.69	1.37	6.04
10°C	Peas	Cotyl.	10.64	4.75	34.20	45.42	4.94	1.40	5.97
10°C	Lima Beans	Cotyl.	30.98	2.42	5.26	52.66	8.69	1.36	2.03
L.S.D.(0.05)			7.91	0.93	5.39	N.S.	0.95	N.S.	3.01
25°C	Broad Beans	Cotyl.	12.98	3.00	37.08	45.30	1.50	1.32	5.27
25°C	Peas	Cotyl.	12.18	4.94	33.08	44.90	4.19	1.35	4.81
25°C	Lima Beans	Cotyl.	34.94	1.83	5.03	49.05	9.11	1.30	1.73
L.S.D.(0.05)			4.08	1.82	7.26	N.S.	1.08	N.S.	0.77

Table 7. Phosphatidyl ethanolamine: Percentages of fatty acids after 24 hours of germination at 10°C or 25°C. Comparison of temperatures and seed parts.

Fatty Acids	Seed part	Broad Beans			Peas			Lima Beans		
		10°C	25°C	L.S.D. (0.05)	10°C	25°C	L.S.D. (0.05)	10°C	25°C	L.S.D. (0.05)
16:0	Axes	12.37	13.33	N.S.	18.03	10.53	4.89	19.17	38.94	7.79
	Cotyl.	12.30	12.98	N.S.	10.64	12.18	N.S.	30.98	34.94	N.S.
L.S.D.(0.05)		N.S.	N.S.		7.96	N.S.		9.44	N.S.	
18:0	Axes	2.34	3.88	N.S.	5.33	3.71	N.S.	2.97	2.52	N.S.
	Cotyl.	2.05	3.00	N.S.	4.75	4.94	N.S.	2.42	1.83	0.47
L.S.D.(0.05)		N.S.	N.S.		N.S.	1.03		N.S.	N.S.	
18:1	Axes	27.60	22.39	N.S.	36.20	38.06	N.S.	16.52	12.61	3.73
	Cotyl.	36.00	37.17	N.S.	34.20	33.08	N.S.	5.26	5.03	N.S.
L.S.D.(0.05		6.17	10.69		N.S.	N.S.		1.28	3.59	
18:2	Axes	54.90	58.33	N.S.	37.23	43.07	N.S.	45.82	28.79	12.51
	Cotyl.	47.96	45.30	N.S.	45.42	44.99	N.S.	52.66	49.05	N.S.
L.S.D.(0.05)		5.29	13.27		N.S.	N.S.		N.S.	12.88	
18:3	Axes	2.77	2.04	0.60	3.17	4.59	N.S.	14.14	17.11	N.S.
	Cotyl.	1.69	1.50	N.S.	4.94	4.19	N.S.	8.69	9.11	N.S.
L.S.D.(0.05)		0.65	0.43		0.97	N.S.		3.33	5.19	
Unsat./Sat.	Axes	6.03	4.90	N.S.	3.35	6.13	2.05	3.50	1.42	1.07
	Cotyl.	6.04	5.27	N.S.	5.97	4.81	N.S.	2.03	1.73	N.S.
L.S.D.(0.05)		N.S.	N.S.		N.S.	N.S.		1.21	N.S.	
Double Bond Index	Axes	1.45	1.45	N.S.	1.20	1.38	0.12	1.50	1.22	0.17
	Cotyl.	1.37	1.32	N.S.	1.40	1.35	N.S.	1.36	1.30	N.S.
L.S.D.(0.05)		N.S.	N.S.		0.19	N.S.		N.S.	N.S.	

had just the opposite (Table 4). All 3 species had a higher percentage 18:3 in axis tissue (Table 4).

(3) 25°C vs. 10°C Imbibition. In general, no significant or consistent differences were seen for any of the fatty acids, for D.B.I. or unsaturated/saturated ratio during imbibition at 25°C vs. 10°C (Table 7).

This may be explained by the fact that the 24 hour imbibition at 25°C or 10°C was a short period of time for large quantities of new phospholipids to be synthesized, containing fatty acids characteristic of the particular temperature. Consequently, the seeds analyzed contained mostly the same fatty acids in their phospholipids as there were at the time of seed maturity. In one of the few exceptions observed, lima bean axes exhibited a higher D.B.I. and unsaturated/saturated ratio at 10°C than at 25°C (Table 7). Apparently the actively growing axis at 25°C had sufficient time to produce quite large quantities of new phospholipids with fatty acids characteristic of 25°C.

(4) Imbibition Stage vs. 6th Day of Germination. No significant changes in the percentages of fatty acids between the imbibition stage and the 6th day of germination, at 10°C, were observed for broad bean and lima bean cotyledons (Table 10). In peas, a decrease in percentage 18:1 and an increase in percentage 18:2 was observed during this 6 day period.

However, in the axes a 10-fold decrease in percentage 18:1 and a 5-fold increase in percentage 18:3 were observed in the chilling resistant species while no significant differences were observed in lima beans at the two stages of germination (Table 8), apparently because of chilling injury and lack of metabolism in this species at 10°C. There were few significant differences between the D.B.I. and unsaturated/saturated ratio which could be related to chilling resistance or sensitivity.

Content of Fatty Acids in Phosphatidyl Inositol

(1) Species Differences.

(a) Large differences were observed in percentage 18:1 and 18:3 in most cases. Chilling resistant species generally exhibited a higher percentage 18:1 and a lower percentage 18:3 than lima beans after 24 hours of imbibition (Tables 8 and 10).

(b) On the 6th day the chilling resistant species maintained the highest percentage 18:1 in cotyledons, while in axes, lima beans had the highest one (Table 3).

Lima beans maintained the highest percentage 18:3 among species after 6 days of germination and they had a higher D.B.I. during the imbibition period than the other species. No significant

Table 8. Phosphatidyl ethanolamine: Percentages of fatty acids after 24 hours or 6 days of germination at 10°C.

Species	Seed part	Germination stage	Fatty Acids 16:0	18:0	18:1	18:2	18:3	Double Bond Index	Unsat./Sat.
Broad Beans	Axes	24th hr.	12.37	2.34	27.64	54.90	2.77	1.45	6.06
	Axes	6th day	18.59	2.92	2.98	64.84	13.15	1.72	3.99
L.S.D.(0.05)			N.S.	N.S.	3.79	N.S.	0.96	N.S.	N.S.
Peas	Axes	24th hr.	18.03	5.33	36.20	37.23	3.17	1.20	3.35
	Axes	6th day	22.43	3.07	3.33	55.74	15.39	1.61	2.97
L.S.D.(0.05)			N.S.	N.S.	3.30	12.90	4.72	0.13	N.S.
Lima Beans	Axes	24th hr.	19.17	2.97	16.52	45.82	14.14	1.50	3.50
	Axes	6th day	25.12	2.48	15.81	40.53	16.04	1.45	2.63
L.S.D.(0.05)			N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Broad Beans	Cotyl.	24th hr.	12.30	2.05	36.00	47.96	1.69	1.37	6.04
	Cotyl.	6th day	13.59	2.71	29.59	51.75	2.31	1.40	5.20
L.S.D.(0.05)			N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Peas	Cotyl.	24th hr.	10.64	4.75	34.20	45.42	4.94	1.40	5.97
	Cotyl.	6th day	15.35	5.64	16.54	58.45	3.97	1.45	3.77
L.S.D.(0.05			N.S.	0.62	7.57	10.37	N.S.	N.S.	N.S.
Lima Beans	Cotyl.	24th hr.	30.98	2.42	5.26	52.66	8.69	1.36	2.03
	Cotyl.	6th day	27.90	2.23	5.78	55.22	8.83	1.42	2.34
L.S.D.(0.05)			N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

Table 9. Phosphatidyl inositol: Percentages of fatty acids after 24 hours of germination at 10°C or 25°C. Comparison of species.

Temp.	Species	Seed part	Fatty Acids 16:0	18:0	18:1	18:2	18:3	Double Bond Index	Unsat./Sat.
10°C	Broad Beans	Axes	30.70	3.39	16.30	46.80	2.82	1.18	1.93
10°C	Peas	Axes	34.78	5.53	21.29	33.45	4.91	1.03	1.48
10°C	Lima Beans	Axes	30.30	7.33	12.05	33.33	17.35	1.30	1.66
L.S.D.().05)			2.47	3.35	4.66	7.49	2.09	0.11	0.25
25°C	Broad Beans	Axes	32.29	6.03	15.05	43.94	2.68	1.11	1.61
25°C	Peas	Axes	33.14	5.99	21.63	34.52	4.67	1.04	1.56
25°C	Lima Beans	Axes	37.78	4.22	8.99	28.63	20.33	1.27	1.38
L.S.D.(0.05)			N.S.	1.19	2.92	4.18	0.71	0.08	N.S.
10°C	Broad Beans	Cotyl.	34.14	3.13	18.43	43.26	1.04	1.08	1.68
10°C	Peas	Cotyl.	29.71	8.70	15.47	40.62	5.47	1.13	1.62
10°C	Lima Beans	Cotyl.	37.46	5.61	3.87	41.73	11.28	1.21	1.33
L.S.D.(0.05)			N.S.	1.83	1.24	N.S.	1.08	N.S.	N.S.
25°C	Broad Beans	Cotyl.	36.60	3.41	16.68	42.08	1.18	1.04	1.49
25°C	Peas	Cotyl.	40.81	7.39	13.51	34.26	3.99	0.94	1.07
25°C	Lima Beans	Cotyl.	36.66	5.69	4.14	42.38	11.11	1.22	1.36
L.S.D.(0.05)			1.76	0.43	1.46	1.29	0.60	0.02	0.08

Table 10. Phosphatidyl inositol: Percentages of fatty acids after 24 hours of germination at 10°C or 25°C. Comparison of temperatures and seed parts.

Fatty Acids	Seed part	Broad Beans 10°C	Broad Beans 25°C	Broad Beans L.S.D. (0.05)	Peas 10°C	Peas 25°C	Peas L.S.D. (0.05)	Lima Beans 10°C	Lima Beans 25°C	Lima Beans L.S.D. (0.05)
16:0	Axes	30.76	32.29	N.S.	34.78	33.14	N.S.	30.30	37.78	4.48
16:0	Cotyl.	34.14	36.60	2.19	29.71	40.81	5.57	37.46	36.66	N.S.
L.S.D.(0.05)		2.73	4.03		N.S.	4.21		6.72	N.S.	
18:0	Axes	3.39	6.03	0.89	5.53	5.99	N.S.	7.33	4.22	1.24
18:0	Cotyl.	3.13	3.41	N.S.	8.70	7.39	N.S.	5.61	5.69	N.S.
L.S.D.(0.05)		N.S.	0.72		N.S.	1.00		N.S.	1.26	
18:1	Axes	16.30	15.05	N.S.	21.29	21.63	N.S.	12.05	8.99	1.80
18:1	Cotyl.	18.43	16.68	N.S.	15.47	13.51	1.03	3.87	4.15	N.S.
L.S.D.(0.05)		N.S.	N.S.		4.86	1.35		0.77	1.94	
18:2	Axes	46.80	43.94	N.S.	33.45	34.52	N.S.	33.33	28.63	4.11
18:2	Cotyl.	43.26	42.08	N.S.	40.62	34.26	5.98	41.73	42.38	N.S.
L.S.D.(0.05)		2.12	N.S.		N.S.	N.S.		3.51	4.14	
18:3	Axes	2.83	2.68	N.S.	4.91	4.67	N.S.	17.23	20.33	2.85
18:3	Cotyl.	1.04	1.18	N.S.	5.47	3.99	1.03	11.28	11.11	N.S.
L.S.D.(0.05)		0.78	0.28		N.S.	N.S.		3.02	0.88	
Unsat./Sat.	Axes	1.93	1.61	0.27	1.48	1.56	N.S.	1.66	1.38	0.27
	Cotyl.	1.68	1.49	0.16	1.62	1.07	0.46	1.33	1.36	N.S.
L.S.D.(0.05)		0.20	N.S.		N.S.	0.29		N.S.	N.S.	
Double Bond Index	Axes	1.18	1.03	0.143	1.03	1.04	N.S.	1.30	1.27	N.S.
	Cotyl.	1.08	1.04	0.038	1.13	0.94	0.14	1.21	1.22	N.S.
L.S.D.(0.05)		0.04	N.S.		N.S.	0.07		N.S.	N.S.	

differences between chilling resistant and sensitive species were observed on the 6th day (Tables 3, 4 and 9).

(2) Axes vs. Cotyledons. During imbibition, there were few consistent differences among species in the content of fatty acids comparing axes and cotyledons. After 6 days germination, broad beans and peas had a higher percentage 18:1 in cotyledons than axis while the opposite was true in lima beans (Table 4). All three species had a higher percentage 18:3 in axis tissue (Table 4).

(3) 25°C vs. 10°C Imbibition. There were only a few significant differences in the percentages of fatty acids in axes or cotyledons in all species, due to temperature during the imbibition stage (Table 10).

(4) Imbibition Stage vs. 6 Day Germination. In axis tissue, there was a decrease in percentage 18:1 and an increase in percentage 18:3 in all three species from the imbibition stage to the 6th day of germination (Table 11). In addition, the percentage 18:2 of broad beans and peas did not change with time, but in lima beans it increased 10-fold (Table 11).

Similar changes with time did not occur in cotyledonary tissue (Table 11).

Table 11. <u>Phosphatidyl inositol</u>: Percentages of fatty acids after 24 hours or 6 days of germination at 10°C.

Species	Seed part	Germination stage	Fatty Acids 16:0	18:0	18:1	18:2	18:3	Double Bond Index	Unsat./Sat.
Broad Beans	Axes	24th hr.	30.76	3.39	16.30	46.80	2.83	1.18	1.93
	Axes	6th day.	37.68	3.45	2.93	46.69	9.20	1.24	1.44
L.S.D.(0.05			5.32	N.S.	3.38	N.S.	1.41	N.S.	0.27
Peas	Axes	24th hr.	34.78	5.53	21.29	33.45	4.91	1.03	1.48
	Axes	6th day	32.13	4.24	4.29	42.39	16.95	1.40	1.75
L.S.D.(0.05)			0.86	N.S.	4.77	N.S.	2.33	0.14	N.S.
Lima Beans	Axes	24th hr.	30.30	7.33	12.05	3.33	17.35	1.30	1.66
	Axes	6th day	30.18	5.55	10.75	31.12	22.37	1.40	1.80
L.S.D.(0.05)			N.S.	0.47	1.02	1.41	4.55	N.S.	N.S.
Broad Beans	Cotyl.	24th hr.	34.14	3.13	18.43	43.26	1.04	1.08	1.68
	Cotyl.	6th day	32.85	3.57	16.81	45.27	1.36	1.11	1.74
L.S.D.(0.05)			N.S.	N.S.	N.S.	1.97	N.S.	N.S.	N.S.
Peas	Cotyl.	24th hr.	29.71	8.70	15.47	40.62	5.47	1.13	1.62
	Cotyl.	6th day	34.74	7.81	12.79	39.81	4.81	1.07	1.35
L.S.D.(0.05)			N.S.	N.S.	1.58	N.S.	N.S.	N.S.	N.S.
Lima Beans	Cotyl.	24th hr.	37.46	5.61	3.87	41.73	11.23	1.21	1.33
	Cotyl.	6th day	36.31	5.86	4.91	42.13	10.74	1.21	1.42
L.S.D.(0.05)			N.S.	N.S.	0.61	N.S.	N.S.	N.S.	N.S.

Discussion

There are many reports in the literature correlating cold hardiness or chilling resistance with highly unsaturated lipids as it was discussed earlier.

Results reported here show that there are large differences in some fatty acids of phospholipids between chilling resistant broad beans and peas and chilling sensitive lima beans.

The fatty acids analyzed in this study were a reflection of total fatty acid content of the total phospholipid present after a 24 hour imbibition of seeds rather than a representation of newly synthesized fatty acids. When the fatty acids of three major phospholipids (PC, PE and PI) were determined in the 3 species studied, there were some very consistent differences.

In all three phospholipids, at 10°C and 25°C, broad beans and peas had a higher percentage 18:1 and a lower percentage 18:3 than lima beans. In PC, lima beans generally had a higher percentage 16:0 than did peas and broad beans in both cotyledon and axis tissue. In PE, lima beans had a higher percentage 16:0 than the other two species in cotyledons but not in axis tissue.

The melting point of 16:0 is 63°C and of 18:1 is about 16°C. The high percentage 16:0 observed in lima beans in PC, and partially in PE, may be related to the transition temperature from liquid crystalline to crystalline state for those phospholipids in the cell membranes. This may contribute to the cold sensitivity of lima beans. On the other hand the fact that lima beans generally had a higher percentage 18:3 (melting point -10°C) than the chilling resistant species is inconsistent with the above concept. Differences in the percentage of 18:0 and 18:2 were not consistently related to the degree of chilling resistance.

The double bond index (D.B.I.) has been used to indicate differences in unsaturation of fatty acids. A high D.B.I. is usually related to cold resistance. The results reported here are contrary to the above in that lima beans had a higher D.B.I. than the chilling resistant species. On the other hand, the unsaturated to saturated raio was higher, especially in cotyledons of the chilling resistant species. This discrepancy is, apparently, because the unsaturated/saturated ratio does not give a higher weight to 18:3 than to 18:1 or 18:2 even though the 18:3 fatty acid is more highly unsaturated and may contribute more to the lowering of the Tc of phospholipids.

The data presented here point out dramatic differences between lima beans, broad beans, and peas in the fatty acid changes which occur over a 6 day germination period at 10°C. In general, in the two major phospholipids (PC and PE), the percentage 18:1 in broad beans and peas decreased from imbibition to 6 days later and the percentage 18:3 increased. This is especially true in axis tissue. This indicates that in these chilling resistant plants, there is a shift in metabolism of fatty acids toward a more highly unsaturated state even though this is not reflected in the D.B.I. or unsaturated/saturated ratio. This again is compatible with the idea that exposure to low temperatures can cause shifts in lipid metabolism of many organisms towards more unsaturated fatty acids. It should be emphasized that even small changes in the overall melting points of the membrane lipids may be crucial for normal functioning at chilling temperatures. Lima beans, which are very sensitive to chilling temperatures, did not exhibit any change in fatty acid composition from imbibition to 6 days later. This probably indicates that the seed was injured by the low temperature and no shift in metabolism was possible because no growth was occurring.

The possibility exists, however, that the changes observed to occur from imbibition to the 6th day at 10°C, could also occur at 25°C. Therefore, definite conclusions cannot be made at this time.

An increase in the desaturation of fatty acids at low temperatures, in a number of plant tissues including seeds (Harris and James, 1969a,b) has been attributed to a higher solubility of oxygen at low temperatures; oxygen is required for the desaturation reactions of fatty acids.

The observed increase in the percentage of 18:3, and sometimes of 18:2, from the imbibition stage to the 6th day of germination (Tables 5, 8 and 11) may be explained according to the above relationship. Accordingly, the majority of fatty acids analyzed in the imbibed seeds apparently were synthesized at the higher temperatures of the period of seed development and consequently under relatively low oxygen concentrations in the cellular environment. On the contrary the majority of the fatty acids analyzed the 6th day of germination, presumably, were synthesized during the six days of germination at 10°C, apparently under higher oxygen concentrations; therefore, they were more unsaturated.

The regulation of the level of the unsaturation of fatty acids by the available oxygen suggested by Harris and James is not known to have any cause-effect relationship with the development of cold hardiness in plants.

SECTION IV

FATTY ACID CONTENT OF PHOSPHOLIPIDS IN SEEDLINGS OF SOME VEGETABLE SPECIES IN RELATION TO CHILLING RESISTANCE

FATTY ACID CONTENT OF PHOSPHOLIPIDS IN SEEDLINGS OF SOME VEGETABLE SPECIES IN RELATION TO CHILLING RESISTANCE

Introduction

Chilling sensitive plants are injured by chilling temperatures, below about 12°C but above 0°C (Lyons, 1973). A partial hardening of those plants against injury from chilling temperatures can be achieved by conditioning them for relatively short periods of time, at temperatures slightly above the chilling range (Wheaton and Morris, 1967; Wilson and Crawford, 1974).

For the reasons presented in the introduction of Sections I and III, it was thought that the analysis of the fatty acids of the major phospholipids (PI, PC and PE) in seedlings of some chilling resistant and chilling sensitive vegetable species, may contribute to the understanding of the mechanism of chilling injury.

The species used to study phospholipid synthesis (Section II) were also used for the analysis of fatty acids of their major phospholipids.

Materials and Methods

Materials

The materials (seeds, chemicals, etc.) described in Section II were employed to conduct the experiments presented in this section.

Methods

The plants analyzed for fatty acids were grown as described in Section II; however they were one day older than those described in Table 1 of that Section. The fatty acid analysis of chilling hardened plants was performed in plants that were maintained for 4 days at 10°C after transfer from 25°C. The plants were frozen in liquid nitrogen, lyophilized and their lipids were extracted and fractionated in individual phospholipids using methods described in Section I.

Methyl ester preparation and GLC analysis as well as calculation of the data was made as described in Section III. The fatty acids analyzed here represent the ones of total PI, PE and PC occurring in the plants at the time of analysis.

Results

(1) Comparison of Species

(a) No correlation between the degree of chilling resistance of the species and the percentage 16:0

could be detected for any of the phospholipids at 25°C or following transfer from 25°C to 10°C (Tables 1, 2 and 3).

(b) In general, the chilling sensitive species had a higher percentage 18:0 than the chilling resistant ones at 25°C and after transfer from 25°C to 10°C (Tables 1 and 2). However, there were some exceptions; peas and cabbage exhibited about the same percentage 18:0 as the chilling sensitive species did. In many comparisons, the differences were not significant.

(c) There were no consistent differences in percentage 18:1, in all phospholipids, between chilling resistant and chilling sensitive species which could be related to sensitivity to chilling injury (Tables 1, 2 and 3).

(d) Chilling resistant species had a higher percentage 18:2 than chilling sensitive ones had, in all phospholipids, at 25°C or after transfer from 25°C to 10°C (Tables 1, 2 and 3).

(e) Chilling sensitive species had a much higher percentage 18:3 than the chilling resistant ones had (with few exceptions), in all phospholipids, at 25°C or after transfer from 25°C to 10°C (Tables 1, 2 and 3).

Table 1. Phosphatidyl Choline: Percentages of fatty acids in seedlings of chilling sensitive and chilling resistant species grown at 10°C, 25°C or after transfer from 25°C to 10°C.

		Chilling resistant species					Chilling sensitive species			
Fatty Acids	Temp.	Broad Beans	Peas	Lettuce	Beets	Cabbage	Lima Beans	Cucum-ber	Water-melon	L.S.D. (0.05)
16:0	10°C	23.20	42.58	24.04	22.70	16.96	--	--	--	6.74
"	25→10°C	29.56	46.19	--	25.05	16.84	35.10	19.69	23.75	4.60
"	25°C	24.12	32.89	26.52	31.89	22.05	40.95	24.72	30.36	5.39
L.S.D.(0.05)		5.24	9.31	N.S.	N.S.	3.71	N.S.	2.69		
18:0	10°C	3.25	6.99	1.75	1.34	0.64	--	--	--	1.85
"	25→10°C	3.74	8.19	--	2.44	1.62	7.54	5.27	8.38	1.26
"	25°C	4.15	11.00	2.26	2.16	3.87	10.56	6.70	8.64	1.32
L.S.D.(0.05)		N.S.	2.81	N.S.	N.S.	0.438	N.S.	0.73	N.S.	
18:1	10°C	1.29	1.48	4.78	3.04	9.92	--	--	--	1.01
"	25→10°C	2.64	3.20	--	8.95	14.33	7.77	0.70	1.65	4.30
"	25°C	2.74	5.27	18.63	6.19	17.24	9.58	1.62	1.65	3.37
L.S.D.(0.05)		0.85	1.50	5.61	N.S.	N.S.	N.S.	0.218	N.S.	
18:2	10°C	64.16	43.83	52.91	71.08	39.12	--	--	--	7.19
"	25→10°C	58.71	40.06	--	63.03	43.87	32.27	19.37	26.66	4.97
"	25°C	64.89	47.53	46.66	58.18	39.37	27.35	19.96	30.89	6.34
L.S.D.(0.05)		N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	2.66	
18:3	10°C	8.05	5.06	16.45	1.75	33.27	--	--	--	2.61
"	25→10°C	5.29	2.28	--	1.78	23.31	17.33	54.93	39.44	2.50
"	25°C	4.06	3.26	5.91	1.54	17.44	11.53	47.02	29.41	3.35
L.S.D.(0.05)		2.14	1.59	2.50	N.S.	4.02	N.S.	4.68	3.33	
	10°C	2.816	1.020	2.876	3.476	5.010	--	--	--	1.504
Unsat./Sat.	25→10°C	2.016	0.843	--	2.730	4.420	1.363	3.016	2.110	0.508
	25°C	2.553	1.316	2.483	1.936	2.870	0.943	2.186	1.590	0.409
L.S.D.(0.05)		N.S.	N.S.	0.402	N.S.	1.031	N.S.	0.449	0.304	
Double	10°C	1.536	1.040	1.600	1.500	1.880	--	--	--	0.148
Bond	25→10°C	1.360	0.900	--	1.380	1.720	1.243	2.043	1.733	0.118
Index	25°	1.446	1.100	1.293	1.273	1.483	0.986	1.826	1.516	0.155
L.S.D.(0.05)		0.127	N.S.	0.119	N.S.	0.099	N.S.	0.087	0.090	

Table 2. Phosphatidyl Ethanolamine: Percentages of fatty acids in seedlings of chilling sensitive and chilling resistant species grown at 10°C, 25°C or after transfer from 25°C to 10°C.

		Chilling resistant species					Chilling sensitive species			
Fatty Acids	Temp.	Broad Beans	Peas	Lettuce	Beets	Cabbage	Lima Beans	Cucumber	Watermelon	L.S.D. (0.05)
16:0	10°C	32.49	41.03	27.57	27.39	17.90	—	—	—	6.41
"	25→10°C	38.93	46.07	28.39	34.10	25.28	41.27	31.61	38.57	5.65
"	25°C	32.98	48.94	28.29	43.70	26.55	42.24	34.23	39.68	4.97
L.S.D.(0.05)		N.S.	N.S.	N.S.	7.59	4.15	N.S.	N.S.	N.S.	
18:0	10°C	2.08	5.26	1.20	1.22	1.24	—	—	—	1.31
"	25→10°C	2.16	4.81	0.83	2.82	3.12	3.09	2.55	3.05	1.48
"	25°C	3.34	5.16	1.53	1.46	2.86	7.03	2.83	3.37	0.76
L.S.D.(0.05)		0.72	N.S.	N.S.	N.S.	N.S.	0.25	N.S.	N.S.	
18:1	10°C	0.34	0.89	2.45	1.22	11.30	—	—	—	0.99
"	25→10°C	0.87	1.51	5.03	4.26	12.45	3.78	0.44	0.52	2.32
"	25°C	1.03	1.54	7.03	3.96	9.72	6.86	0.83	0.51	1.19
L.S.D.(0.05)		0.54	N.S.	1.23	1.55	N.S.	2.39	N.S.	N.S.	
18:2	10°C	58.64	46.86	53.14	69.32	40.12	—	—	—	6.07
"	25→10°C	55.81	44.32	58.13	56.93	40.54	35.93	18.13	21.53	4.65
"	25°	58.40	42.12	57.59	50.44	42.56	33.48	18.83	25.72	4.40
L.S.D.(0.05)		N.S.	N.S.	2.95	7.11	N.S.	N.S.	N.S.	2.19	
18:3	10°C	6.42	5.95	15.60	1.29	29.73	—	—	—	2.07
"	25→10°C	3.47	3.22	7.66	1.84	18.62	15.93	47.19	36.26	2.25
"	25°C	4.21	2.20	5.52	0.35	18.27	10.40	43.33	29.69	2.46
L.S.D.(0.05)		N.S.	N.S.	1.37	1.09	2.08	4.37	N.S.	2.07	
Unsat./Sat.	10°C	1.916	1.213	2.500	2.596	4.246	—	—	—	0.725
	25→10°C	1.500	0.966	2.413	1.730	2.523	1.286	1.926	1.406	0.334
	25°C	1.780	0.860	2.356	1.213	2.430	1.023	1.706	1.926	0.378
L.S.D.(0.05)		N.S.	0.264	N.S.	0.878	0.571	N.S.	N.S.	N.S.	
Double Bond Index	10°C	1.390	1.123	1.553	1.430	1.810	—	—	—	0.145
	25→10°C	1.233	1.000	1.433	1.236	1.490	1.233	1.780	1.520	0.132
	25°C	1.306	0.923	1.386	1.056	1.493	1.046	1.683	1.410	0.122
L.S.D.(0.05)		N.S.	N.S.	0.079	0.176	0.107	N.S.	N.S.	0.064	

Table 3. Phosphatidyl Inositol: Percentages of fatty acids in seedlings of chilling sensitive and chilling resistant species grown at 10°C, 25°C or after transfer from 25°C to 10°C.

		Chilling resistant species					Chilling sensitive species			
Fatty Acids	Temp.	Broad Beans	Peas	Lettuce	Beets	Cabbage	Lima Beans	Cucumber	Watermelon	L.S.D. (0.05)
16:0	10°C	42.62	51.38	52.29	39.74	46.67	—	—	—	8.24
"	25→10°C	35.65	56.28	40.64	37.95	50.30	46.00	35.11	38.12	5.40
"	25°C	39.14	53.53	52.75	53.17	44.28	49.83	41.06	44.15	4.29
L.S.D.(0.05)		N.S.	N.S.	1.04	6.79	N.S.	N.S.	5.46	3.68	
18:0	10°C	2.69	3.93	2.46	4.32	3.28	—	—	—	N.S.
"	25→10°C	2.24	5.19	3.95	6.21	3.29	5.55	4.91	9.11	3.04
"	25°C	3.38	6.00	2.90	5.33	4.27	7.95	4.01	7.02	1.77
L.S.D.(0.05)		0.46	N.S.	0.90	N.S.	N.S.	N.S.	N.S.	N.S.	
18:1	10°C	1.08	0.81	0.95	3.12	3.26	—	—	—	0.90
"	25→10°C	1.87	5.68	4.70	7.51	4.23	3.98	1.09	3.93	N.S.
"	25°	1.76	2.88	2.22	2.53	7.32	4.56	1.07	1.29	1.69
L.S.D.(0.05)		0.64	N.S.	1.08	2.32	1.74	N.S.	N.S.	N.S.	
18:2	10°C	45.79	35.18	41.43	47.98	22.66	—	—	—	6.35
"	25→10°C	53.58	29.89	46.85	44.83	25.88	25.29	11.29	15.02	3.47
"	25°	51.67	34.39	39.90	36.84	25.89	23.54	11.73	18.93	5.31
L.S.D.(0.05)		N.S.	N.S.	1.93	N.S.	N.S.	N.S.	N.S.	2.79	
18:3	10°C	7.84	8.70	2.84	5.05	24.07	—	—	—	3.17
"	25→10°C	6.61	3.15	3.80	3.51	16.43	19.17	47.58	33.75	6.05
"	25°	4.24	3.29	2.43	3.04	18.14	14.10	42.16	28.80	1.74
L.S.D.(0.05)		1.22	2.66	N.S.	N.S.	2.34	3.96	4.44	N.S.	
Unsat./Sat.	10°C	1.229	0.823	0.826	1.270	1.013	—	—	—	0.285
	25→10°C	1.666	0.630	1.243	1.270	0.863	0.940	1.503	1.126	0.267
	25°	1.363	0.683	0.798	0.713	1.070	0.730	1.223	0.960	0.143
L.S.D.(0.05)		N.S.	N.S.	0.034	0.216	N.S.	0.168	0.239	N.S.	
Double Bond Index	10°C	1.160	0.976	0.920	1.140	1.206	—	—	—	0.155
	25→10°C	1.290	0.753	1.100	1.076	1.056	1.120	1.666	1.350	0.159
	25°	1.363	0.813	0.882	0.820	1.136	0.940	1.506	1.253	0.087
L.S.D.(0.05)		N.S.	0.169	0.019	0.103	N.S.	0.120	0.111	N.S.	

(f) The ratio of unsaturated/saturated fatty acids, in all phospholipids, did not correlate well with the known differences in chilling sensitivity between species (Tables 1, 2 and 3).

(g) The chilling sensitive species exhibited a higher D.B.I. than the chilling resistant ones in most of the cases (Tables 1, 2 and 3).

(2) Temperature Comparisons

(a) In most of the species no significant change or a decrease in percentage 16:0 was observed when the temperature was lowered from 25°C to 10°C. The same was true in chilling resistant species when the 25°C and 10°C treatments were compared (Tables 1, 2 and 3).

(b) The percentage of 18:0 either did not change with temperature or it was lower in plants growing continuously at 10°C or transferred from 25°C to 10°C (Tables 1, 2 and 3).

(c) In general, the percentage 18:1 followed a similar pattern, when comparing temperature treatments within species, as shown for 16:0 and 18:0 (Tables 1, 2 and 3).

(d) No consistent differences in percentage 18:2 due to temperature treatment were detected in any of the species (Tables 1, 2 and 3).

(e) In most of the cases the percentage 18:3 was higher at 10°C than at 25°C, in both chilling resistant and sensitive species (Tables 1, 2 and 3).

(f) In general, the unsaturated/saturated fatty acid ratio remained the same or increased upon exposure to low temperatures for all the species.

(g) The D.B.I. remained constant or increased in all species upon exposure to low temperatures.

Discussion

More unsaturated phospholipids have been found in micro-organisms (Cronan and Vagelos, 1972; Kates and Paradis, 1973; Kaneda, 1972) and plants (Grenier and Willemot, 1974; De La Roche *et al.*, 1972) grown at lower temperatures; in cold resistant varieties of alfalfa vs. cold sensitive ones (Grenier and Willemot, 1974); in chilling hardened plants of *Phaseolus vulgaris* and *Gossypium hirsutum* vs. non-hardened ones (Wilson and Crawford, 1974).

On the other hand in alfalfa (Kuiper, 1970) the unsaturation of the fatty acids of phospholipids was not different in plants grown at low or high temperatures or in varieties differing in cold hardiness.

It is generally believed that chilling or cold resistance is associated with highly unsaturated membrane lipids (Lyons *et al.*, 1964; Lyons, 1973).

The results of this study support, with some exceptions, the findings of others about the relationship of fatty acid unsaturation and chilling resistance. In general, the chilling resistant species had a lower percentage 18:0 and 18:3 and a higher percentage 18:2 than did the chilling sensitive ones. The differences in 18:0 and 18:2 between sensitive and resistant species appears to be consistent with the expected higher degree of unsaturation in resistant species. However, the high level of 18:3 in chilling sensitive species was unexpected and difficult to explain.

The unsaturated/saturated ratio did not correlate well with chilling sensitivity and the D.B.I. was actually higher in chilling sensitive species, which is opposite of what was expected.

Exposure of all species to low temperature resulted in a decrease in 16:0, 18:0, 18:1 and in an increase in 18:3, unsaturated/saturated ratio and D.B.I. These results support the findings of others which generally show an increase in unsaturation upon exposure to low temperatures. Few differences in the fatty acid content, in the chilling hardened plants, among chilling resistant and chilling sensitive species were observed. This was, probably, due to the fact that chilling sensitive species in the seedling stage are able to modify their fatty acid metabolism, in response to low temperatures, towards forms similar to the ones observed in chilling resistant species; this modification may contribute to the relative chilling resistance of the lima bean seedling as opposed to the chilling sensitivity of the germinating lima bean seed (Section III).

Furthermore, there is a possibility that the observed higher percentage of 18:3 at 10°C or after transfer from 25°C to 10°C in comparison to 25°C, can be explained according to the findings of Harris and James (1969a,b) as it has been discussed in Section III).

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Significant differences in phospholipid synthesis were observed between chilling resistant and chilling sensitive species as well as between low and high temperatures. In general, PC and to a lesser extent PG, was shown to correlate positively with chilling resistance or growth at low temperatures. The opposite was shown for PE and to a lesser extent for PI.

(1) Specifically, the chilling resistant species (broad beans and peas) during the imbibition stage, at 10°C and 25°C, exhibited a much higher PC/PE ratio than the chilling sensitive species lima beans. In general, chilling resistant species, during imbibition, exhibited a higher percentage PG and PC and a lower percentage PE and PI at 10°C than at 25°C. An apparent shift in phospholipid metabolism towards PE and PG, largely at the expense of PC and PI, was observed in imbibed lima bean seeds at 10°C in comparison to 25°C.

(2) A higher percentage of PG, and in some cases of PC, and a lower percentage of PE was observed at 10°C than at 25°C in seedlings of chilling resistant species.

Transferring chilling resistant and sensitive plants from 25°C to 10°C generally caused an increase in percentage PC and PG and a decrease in percentage PE. It is suggested that lima beans, in the seedling stage, have the ability to shift phospholipid metabolism towards a preferential synthesis of PC at the expense of PE when plants are transferred from 25 to 10°C and probably avoiding chilling injury for that reason, while they are unable to do so at the imbibition stage.

In general, a higher ratio PC/PE was observed at lower temperatures than at higher ones. Generally, the percentage PG was higher in chilling resistant species vs. chilling sensitive ones but other phospholipids could not be related to the degree of chilling resistance of the species.

The observed differences in phospholipid synthesis may be related to the degree of chilling resistance in the species studied since the physical properties of individual phospholipids in relation to temperature are different.

Preferential synthesis of some phospholipid classes by chilling resistant plants or by both chilling resistant and sensitive plants when they are exposed to chilling temperatures, may provide the plant cells with the ability to build membranes which have a specific physical state suitable for normal functioning at low temperatures. On the other hand, it is also possible that the differences in phospholipid synthesis observed in this study have no cause-effect relationship with cold resistance but may represent genetic differences or temperature responses related to other functions of plant metabolism.

Much more research is required to elucidate the possible relationship of chilling or cold resistance to phospholipids. Quantitative and qualitative phospholipid analyses, coupled with physical measurements *in vivo* and *in vitro*, in membrane systems (e.g. mitochondria, chloroplasts, plasmalemma, etc.) will be useful to study the problem. The discovery of mutants, unable to synthesize some of the phospholipid classes, may help to investigate the importance of each phospholipid class in relation to temperature.

(3) The analysis for fatty acid content in PI, PC and PE in germinating chilling resistant and sensitive species revealed considerable differences. In general, broad beans and peas had a higher percentage 18:1 and a lower percentage 18:3 and 16:0 than lima beans. Considering the melting point of those fatty acids (presumably this affects the temperatures at which the phospholipids change phase from a liquid crystalline to a crystalline state) it appears that the chilling sensitive lima beans exhibited an unexpected high percentage 18:3 and D.B.I. However, the unsaturated/saturated ratio was higher in the chilling resistant species as was expected. During the

6 day germination period for chilling resistant species, the percentage 18:1 decreased and 18:3 increased indicating a shift of fatty acid metabolism towards more unsaturated forms. This shift may be a response to low temperatures or a characteristic of the chilling resistant species. However, it is possible that the shift is also observed at high temperature both in chilling resistant and sensitive species. In the chilling sensitive lima beans no changes were observed in individual fatty acids between the imbibition stage and the 6th day of germination presumably because of the absence of normal metabolism at 10°C. It should be pointed out, however, that the exposure of lima beans to chilling temperatures did not result in a break down of phospholipids since they maintained about the same fatty acid content during the six day chilling.

(4) In seedlings grown continuously at 25°C or temperature conditioned at 10°C, the chilling resistant species generally had a higher percentage 18:2 and a lower percentage 18:0 and 18:3 than chilling sensitive species.

Chilling sensitive species, unexpectedly, had a higher D.B.I. than chilling resistant ones, while the ratio of unsaturated/saturated was not correlated with chilling sensitivity of the species. In general, the percentage 18:3 was higher at 10°C than at 25°C or after transferring from 25°C to 10°C, in chilling resistant species.

Exposure of all species to low temperatures generally resulted in a decrease in 16:0, 18:0, 18:1 and an increase in 18:3, unsaturated/saturated ratio and D.B.I. These results support the findings of other workers which show an increase in unsaturation upon exposure to low temperature.

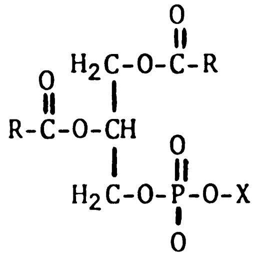
The lack of a consistently higher unsaturated/saturated ratio and D.B.I. in chilling resistant species vs. chilling sensitive species may suggest that the degree of unsaturation of cellular lipids is not the most important factor determining the chilling resistance of plants. The same suggestion which minimizes the importance of fatty acid unsaturation in determining cold sensitivity has been made earlier by Lyons (1973) but it cannot be proven on the basis of the knowledge presently available. In *E. coli* (Esfahani *et al.*, 1971) and sheep liver mitochondria (Lyons, 1973) the fatty acid composition of lipids determined the transition temperature (Tc) of the lipid portion of the respective membranes. In contrast to the above, it has been found, that dietary modifications altered the fatty acid composition of lipids in mitochondrial membranes of rat liver but no considerable shifting of Tc was observed (Williams *et al.*, 1972); there is no similar information available for higher plants.

The results of Section I and II suggest that the polar groups of cellular phospholipids are important in determining the degree of chilling resistance of the species studied as well as the development of chilling hardiness.

APPENDIX

Table A-1. Chemical formulas of the major plant phospholipids.

GENERAL FORMULA OF PHOSPHCLIPIDS



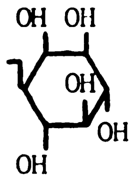
Phosphatidyl-Choline	$X = -CH_2-CH_2-N^+(CH_3)_3$
Phosphatidyl-Ethanolamine	$X = -CH_2-CH_2-NH_2$
Phosphatidyl-Serine	$X = -CH_2-\overset{NH_2}{\overset{\mid}{CH}}-COOH$
Phosphatidyl-Glycerol	$X = -CH_2-\underset{OH}{\underset{\mid}{CH}}-CH_2OH$
Phosphatidyl-Inositol	X = 

Table A-2. Melting point of some fatty acids.*

Carbon Atoms & Double Bonds	Common Name	Systematic Name	Melting Point °C
16:0	Palmitic	n-Hexadecanoic	63:0
18:0	Stearic	n-Octadecanoic	69.6
18:1	Oleic	cis-9-Octadecenoic	14.8
18:2	α-Linoleic	cis-9, cis-12-octadecadienoic	- 5.0
18:3	α-Linolenic	cis-9, cis-12, cis-15-octadecatrienoic	-10.6

*From: Handbook of Biochemistry, 1968. Published by the Chemical Rubber Co., U.S.A.

Table A-3. Approximate transition temperature (Tc), from liquid-crystalline to crystalline state, of some phospholipids.*

Phospholipid	Fatty Acid Chains	Tc (°C)
2,3-dipalmitoyl-DL-1-phosphatidyl-Ethanolamine, Anhydrous	16:0	123[a]
2,3-dipalmitoyl-DL-1-phosphatidyl-Choline, Anhydrous	16:0	94[a]
2,3-dipalmitoyl-DL-1-phosphatidyl-Ethanolamine, in Water	16:0	86[a]
2,3-dipalmitoyl-DL-1-phosphatidyl-Choline, in Water	16:0	44[a]
2,3-distearoyl-DL-1-phosphatidyl-Ethanolamine, Anhydrous	18:0	126[a]
2,3-distearoyl-DL-1-phosphatidyl-Choline, Anhydrous	18:0	101[a]
2,3-distearoyl-DL-1-phosphatidyl-Ethanolamine, in Water	18:0	91[a]
2,3-distearoyl-DL-1-phosphatidyl-Choline, in Water	18:0	62[a]
2-oleoyl-3-stearoyl-DL-1-phosphatidyl-Ethanolamine, Anhydrous	18:0&18:1	72[b]
2-oleoyl-3-stearoyl-DL-1-phosphatidyl-Choline, Anhydrous	18:0&18:1	36[b]
Egg yolk lecithin (Mixture of natural Phosphatidyl Cholines)		22[b]

*From: (a) Williams and Chapman, 1970; (b) Chapman, 1967.

Table A-4. Distribution of radioactivity in lipids of seeds imbibed for 24 hours with ^{14}C-glycerol.

Seed Part	Species	Temperature °C	Specific activity mCi/m mole	Incubation medium µCi/ml	Total lipid CPM(X10^{-3}) per gram dry weight	Radioactivity in phospholipids: CPM (X10^{-3}) per gram dry weight							
						PA	PS	LPC	PI	PC	PE	PG	CL
Axes	Broad Beans	10°C	121	0.2	438	26	--	--	17	136	61	24	--
		25°C	133.4	0.2	234	0.8	--	--	17	105	38	11	--
	Peas	10°C	121	0.2	1,354	161	--	--	77	408	153	73	--
		25°C	121	0.2	366	19	5	--	39	127	54	17	3
	Lima Beans	10°C	121	0.2	870	10	0.2	0.5	27	200	185	92	2
		25°C	133.4	0.2	1,584	6	50	--	159	1,169	583	127	8
Cotyledons	Broad Beans	10°C	121	0.2	200	9	--	--	15	81	19	13	--
		25°C	133.4	0.2	107	--	--	--	8	50	15	3	--
	Peas	10°C	121	0.2	145	1	--	--	12	60	19	6	--
		25°C	121	0.2	24	--	--	--	3	10	3	1	--
	Lima Beans	10°C	121	0.2	24	1	--	--	1	4	6	5	--
		25°C	133.4	0.2	103	--	--	--	12	38	24	4	--

Table A-5. Distribution of radioactivity among lipids of seedlings after a 24 hour incubation with ^{14}C-glycerol.

Species	Temperature °C	Specific activity mCi/m mole	Incubation medium µCi/ml	Total lipid CPM(X10-3) per gram dry weight	Radioactivity in phospholipids: CPM ($X10^{-3}$) per gram dry weight							
					PA	PS	LPC	PI	PC	PE	PG	CL
Broad Beans	10°C	--	--	--	--	--	--	--	--	--	--	--
	25→10°C	7.4	0.5	2,062	247	3	--	169	740	131	226	1
	25°C	7.4	0.5	802	102	2	--	62	249	105	45	--
Peas	10°C	7.4	0.5	1,684	527	3	11	135	265	122	147	1
	25→10°C	7.4	0.5	1,233	328	3	---	125	264	75	122	--
	25°C	7.4	0.5	1,286	315	9	7	125	334	173	101	4
Lettuce	10°C	7.4	0.5	4,491	654	7	--	314	904	634	548	2
	25→10°C	7.4	0.5	12,336	1,024	--	--	740	3,349	1,742	1,284	--
	25°C	7.4	0.5	4,592	1,646	20	--	257	186	510	149	4
Beets	10°C	7.4	0.5	3,611	994	--	--	163	368	654	357	--
	25→10°C	7.4	0.5	4,394	932	3	--	283	620	601	431	--
	25°C	7.4	0.5	2,416	643	--	--	138	978	440	160	1
Cabbage	10°C	7.4	0.5	8,416	1,378	11	--	481	1,591	1,271	1,132	--
	25→10°C	7.4	0.5	3,826	912	3	--	247	467	407	322	--
	25°C	7.4	0.5	2,420	471	11	--	106	293	398	207	--
Lima Beans	10°C	--	--	--	--	--	--	--	--	--	--	--
	25→10°C	7.4	0.5	1,788	327	2	17	183	378	109	90	--
	25°C	7.4	0.5	2,630	509	9	11	263	461	311	175	1
Cucumber	10°C	--	--	--	—	--	--	--	--	--	--	--
	25→10°C	121	0.5	5,974	506	12	--	847	1,625	750	597	2
	25°C	133.4	0.5	7,935	75	--	--	390	1,785	3,265	655	--
Water-melon	10°C	—	--	--	--	--	--	--	--	--	--	--
	25 10°C	7.4	0.5	3,416	1,536	7	12	317	626	348	189	--
	25°C	7.4	0.5	3,061	1,154	3	13	209	344	354	112	--

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